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PRINCIPAL INVESTIGATOR: Robert R. Clarke, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20007

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Robert R. Clarke, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20007

E-Mail: clarker@georgetown.edu

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The factors driving resistance to antiestrogens are unknown. Comparing the transcriptomes of antiestrogen responsive and resistant MCF-7 variants by serial analysis of gene expression, we have implicated several genes, including the human X-box binding protein-1 (XBP-1). XBP-1 is a cAMP response element (CRE) biding protein associated with estrogen receptor (ER) expression in gene expression profiles of human breast cancers. **We hypothesize** that overexpression of XBP-1 and/or activation of CRE contribute functionally to the ability of responsive cells to survive the metabolic stresses induced by exposure to antiestrogens. **We also hypothesize** that measuring expression of the XBP-1 protein will assist in better identifying antiestrogen resistant and/or responsive tumors. **Aim 1:** We will further study the likely functional role of XBP-1/CRE by overexpression through transfection into responsive cells, and inhibiting expression in resistant cells using novel CRE oligonucleotide decoys, antisense and/or ribozymes. Effects of these molecular manipulations on responsiveness to antiestrogens will be studied *in vitro* and *in vivo*. **Aim 2:** We will explore the prognostic and predictive significance of XBP-1 expression in a unique series of human breast cancer biopsies. Thus, we will assess the extent to which XBP-1 is an independent prognostic factor, and whether it is associated with response to antiestrogens. Where possible, we will explore whether XBP-1 expression data allow us to build better predictive/ prognostic models.

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1. Gu, Z., Lee, Y.R., Skaar, T.C., Bouker, K.B., Welch, J.N., Lu, J., Liu, A., Davis, N., Wang, Y. & Clarke, R. "Association of interferon regulatory factor-1, nucleophosmin, nuclear factor-κB and cAMP response element binding with acquired resistance to Faslodex (ICI 182,780)." *Cancer Res,* 8: 1155-1166, 2002.

2. Clarke, R., Liu, M.C., Bouker, K.B., Gu, Z., Lee, R.Y., Zhu, Y., Skaar, T.C., Gomez, B., O'Brien, K., Wang, Y., Hilakivi_Clarke, L.A. "Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling." *Oncogene,* 22: 7316-7339, 2003.

INTRODUCTION

Antiestrogens are effective in premenopausal and postmenopausal patients, and in the chemopreventive, adjuvant and metastatic settings (5), probably through the induction of growth arrest/apoptosis (5). The triphenylethylene TAM, a partial agonist, is the most widely used antiestrogen. Long term TAM use reduces the incidence of contralateral breast cancer (antagonist) and primary breast cancer in high risk women (antagonist), maintains bone density (agonist) and increases the risk of endometrial carcinomas (agonist) (10). Newer antiestrogens include the "pure antagonist" ICI 182,780 (no agonist activity). In patients that had previously shown a response to TAM but recurred, ICI 182,780 produces a response rate significantly higher than the response rate for crossover to another triphenylethylene (Toremifene) following TAM failure (29).

Antiestrogen Resistance. Most breast tumors that initially respond to TAM recur and require other endocrine or cytotoxic therapies (9). Despite over 10 million patient years of experience with TAM, the precise mechanisms that confer acquired resistance are unknown (5). Absence of ER expression is clearly important for *de novo* resistance (5). ER expression is *not* lost in most breast tumors that acquire antiestrogen resistance (15). Currently, there is little compelling evidence that expression of ER splice variants and mutant ER contribute significantly to antiestrogen resistance in patients (5,16). While the importance of wild type ERα is established as a mediator/predictor of antiestrogen responsiveness, that of ERβ remains unclear. ERα may be the predominant species in most ER+ breast tumors (20,26), and is associated with a better prognosis (11). ERβ is associated with a poorer prognosis, absence of PgR, and lymph node involvement (8,26). One small study reported higher ERβ mRNA levels in resistant tumors (25). However, this association could not be separated from that between ER and a more aggressive phenotype (8,26). Some studies report activities independent of ER function, which may initiate events that are necessary but not sufficient for antiestrogen-induced effects (5). Our research team has recently reviewed in detail the potential mechanisms of antiestrogen resistance in ER+ tumors (6).

Our Models of Antiestrogen Resistance. We generated and characterized a series of variants from an E2-independent variant of MCF-7 cells (MCF7/MIII), selected for growth in nude mice (4). Further *in vivo* selection produced MCF7/LCC1 cells (2), which also retain ER expression, are E2-independent for growth, and are inhibited by antiestrogens (2,3). Subsequently, MCF7/LCC1 cells were stepwise selected against increasing concentrations of 4OH-TAM or ICI 182,780 *in vitro*. Cells selected against 4OH-TAM produced stable, TAM resistant cells (MCF7/LCC2) that are not crossresistant to ICI 182,780 (Faslodex) (3), predicting that tumors responding and then failing TAM might respond to Faslodex (3). Confirming the clinical relevance of the MCF7/LCC2 phenotype, patients with *acquired* TAM resistance responded upon crossover to ICI 182,780 with a response rate higher than with crossover to other triphenylethylenes (14). Cells resistant to ICI 182,780 (MCF7/ LCC9) were generated by selecting MCF7/LCC1 cells against ICI 182,780. MCF7/LCC9 cells are ER+, ICI 182,780 resistant, and TAM crossresistant. TAM crossresistance emerges early during selection, and before stable ICI 182,780 resistance (3). Comparing MCF7/LCC9 and MCF7/LCC2 cells, we can identify mechanisms of resistance to TAM that may/may not confer crossresistance to ICI 182,780.

Implicating XBP-1 in Antiestrogen Resistance.

SAGE. Initially, we explored differences in the transcriptomes of the MCF7/LCC1 and MCF7/LCC9 cells by serial analysis of gene expression (SAGE) as previously described (28), using the "SAGE" software (Dr. Kinzler, Johns Hopkins University). Most genes identified are not differentially expressed between MCF7/LCC1 and MCF7/LCC9 cells. Differentially expressed genes were selected by (a) the Tags compared represent ≤2 genes, (b) a Tag found in either the MCF7/LCC1 or MCF7/LCC9 SAGE library must represent 0.10% of the database, and (c) fold difference ~2-fold. Evidence that a gene is expressed in breast cancers also was considered. No single criterion was considered an absolute requirement for selection. Among the genes we identified were cathepsin D, nucleophosmin (NPM) and XBP-1. We have shown NPM to be induced by E2 in MCF-7 cells, upregulated in MCF7/LCC1 cells (24) and further upregulated (~2-fold by Western) in MCF7/LCC9 cells (not shown), and to provoke an autoimmune response in breast cancer patients associated with TAM therapy (1). Altered expression of cathepsin D is consistent with its increased secretion in our E2-independent MCF-7 variants (27).

Altered expression of XBP-1 protein and transcriptional activation (CRE). To confirm the altered expression of XBP-1, we first performed Western analysis on proteins from MCF7/LCC1 and MCF7/LCC9 cells. We initially detected a ~5-fold induction of XBP-1 protein in MCF7/LCC9 cells, comparable with the 4-fold induction in mRNA levels (12). Measuring protein levels and/or protein bound to responsive elements can be poor indicators of the functional activation of transcription factors. Since XBP-1 activates CREs, we measured directly CRE transcriptional activation using a CRE promoter-firefly luciferase reporter assay (PathDetect *in vivo* signal transduction pathway *cis*-reporting system; Stratagene). Cells were transiently transfected with the appropriate plasmids using Qiagen's Superfect reagent. Normalization of transfection efficiency was made to a *Renilla* luciferase reporter driven by the constitutive cytomegalovirus promoter (Promega's Dual-luciferase reporter assay). The data in Fig. 1 show that basal CRE activity is significantly increased in MCF7/LCC9 cells compared with MCF7/LCC1 cells (4-fold; $p<0.02$).

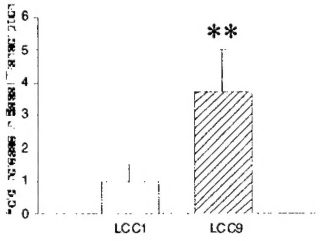

Fig 1: *Transcriptional activation of CRE is increased 4-fold in MCF7/LCC9 cells. **$p<0.02$, $n=6$.*

Regulation of CRE (XBP-1) activity by ICI 182,780. The upregulation of CRE activation would be of limited use to cells if it could be inhibited by ICI 182,780-occupied ERs. Thus, we assessed the ability of ICI 182,780 to affect CRE activation using the promoter-reporter assay. ICI 182,780 treatments (10 nM) were administered for 48 hrs post-transfection. ICI 182,780 treatment does not alter the transcriptional regulatory activities of the CRE promoter in either responsive MCF7/LCC1 or resistant MCF7/LCC9 cells (Fig 2). These data further imply a functional role for XBP-1 in acquired resistance to ICI 182,780. In responsive cells, the inability to induce CRE in the presence of ICI 182,780 allows for the dominance of growth inhibitory signals leading to growth arrest/apoptosis. Resistant cells may survive growth inhibition/apoptosis by upregulating signaling through CREs. ***Since CRE-activation is required for MCF-7 cell proliferation (19), some breast cancer cells may survive antiestrogen treatment by upregulating factors that are not affected by ER-mediated signaling, e.g., XBP-***

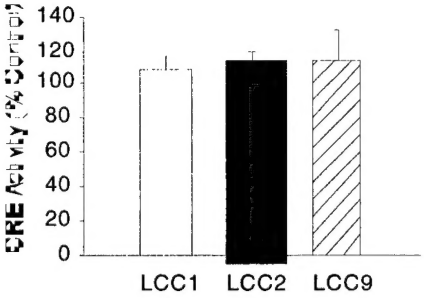

Fig 2: *ICI 182,780 does not regulate CRE activity in either MCF7/LCC1 or MCF7/LCC9 cells. Not significantly different from controls (activity in untreated cells for each variant).*

1/CRE.

BODY OF REPORT

KEY RESEARCH ACCOMPLISHMENTS

We have made good progress on Aim 1 but are not quite where we had anticipated. In contrast, we have moved ahead rapidly on addressing Task 4. Thus, we are probably ahead of where we might have expected to be overall.

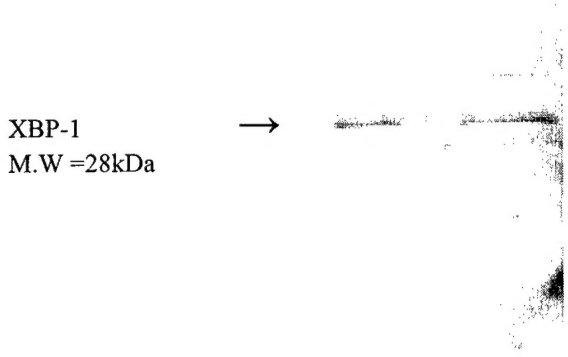

Fig. 3. Western blot of two MCF-7/XBP-1 clones expressing the XBP-1 protein.

TASK 1: Overexpress XBP-1 in antiestrogen sensitive cells

a. Transfect and select transfectants (months 1-2)
b. Characterize transfectants for XBP-1 protein expression and CRE transcriptional activity (months 3-4)
c. Determine response of transfectants and controls to E2 and antiestrogens *in vitro* (months 4-8)
d. Determine response of transfectants and controls to E2 and antiestrogens *in vivo* (months 8-12)

Transfection of MCF-7 cells with XBP-1. We first introduced the XBP-1 cDNA into MCF7 cells, which are antiestrogen sensitive and the parental cell line of LCC1, LCC2 and LCC9 cells. Cells were transfected with a pcDNA 3.1 expression vector (Invitrogen) containing the XBP-1 cDNA. The empty vector (same construct but without the XBP-1 cDNA) also was transfected into MCF-7 cells to generate control cell populations. We had some difficulty getting XBP-1 overexpressed, the reasons for which are not yet apparent. Nonetheless, after several attempts, we successfully obtained clones resistant to G418 (selectable marker).

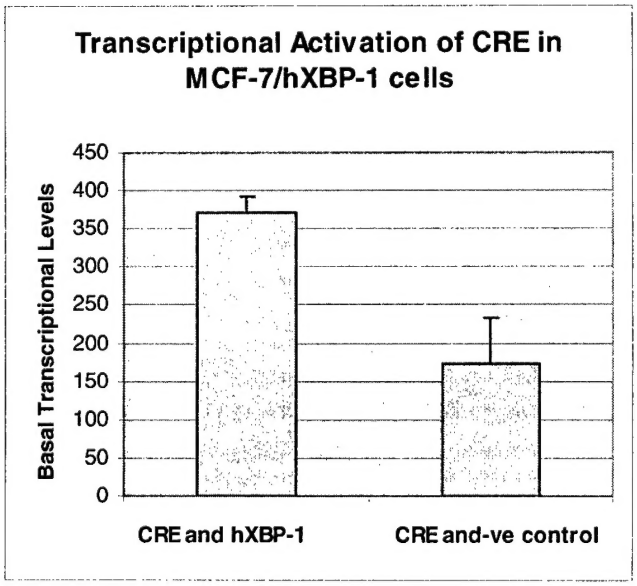

Fig 4: XBP-1 transcriptional activation in transfectants.

Confirmation of XBP-1 protein overexpression and activity. We used western blotting and a commercial antibody against XBP-1 (Santa Cruz), and confirmed overexpression of XBP-1 protein (Fig 3). XBP-1 is a nuclear transcription factor that regulates gene transcription through its ability to active CREs (7). Thus, we next confirmed the increased activity of XBP-1 using a CRE-based promoter-reporter assay Promega's Dual-luciferase reporter assay), as we have previously described (12). The data in Fig 4 show that we successfully increased basal XBP-1 activity by

approximately 2-fold in MCF-7 cells ($p<0.05$). We are currently selecting T47D cells transfected with the XBP-1 cDNA, and will likely attempt a further transfection into MCF-7 cells in the hope of obtaining higher levels of expression.

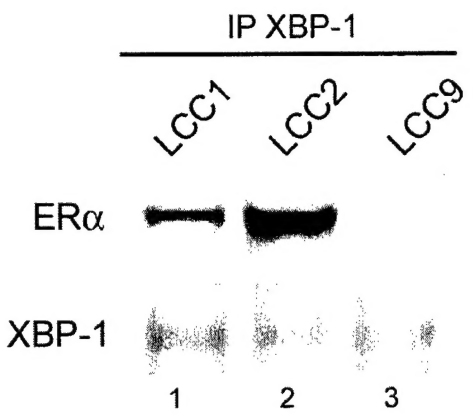

Fig. 5. ERα western blot of XBP-1 immunoprecipitates from LCC1, LCC2 and LCC9 cells.

XBP-1 binds directly to the estrogen receptor. While not part of our statement of work, we used the antibodies from Task 4 to immunoprecipitate XBP-1 from within MCF-7 cells, and then use a western blotting with the ERα antibody (Fig 3). This is an initial study and is currently being repeated. As with all immunoprecipitations and westerns, quantitation of signals is difficult. Nonetheless, immunoprecipitated XBP-1 brings down ERα, clearly suggesting that XBP-1 may be a regulator of ERα function. We do not yet know if the changes in XBP-1/ERα associations in the different cell lines are consistently observed.

TASK 2: Inhibit XBP-1 expression in antiestrogen resistant cells

a. Determine ability of CRE decoys to affect CRE activity and response to E2 and antiestrogens *in vitro* (months 13-14)
b. Determine ability of antisense oligos to affect XBP-1 expression, CRE activity, and response to E2 and antiestrogens *in vitro* (months 14-16)
c. Select active ribozyme from ribozyme library and transfect into MCF7/LCC9 cells (months 17-19)
d. Determine ability of ribozyme transfectants to affect XBP-1 expression, CRE activity, and response to E2 and antiestrogens *in vitro* (months 19-21)
e. Determine the ability of decoys, antisense or oligos to affect response to E2 and antiestrogens *in vivo* (months 21-24)

We did not propose to start these studies in year 1.

TASK 3: Timing of acquired increase in XBP-1 expression and CRE activation

a. Thaw and expand four passages from selection of MCF7/LCC1 to MCF7/LCC9 cells (month 25)
b. Study expression of XBP-1 protein by Western (month 26)
c. Study CRE activation by promoter-reporter assay (month 27)
d. Study XBP-1 expression and CRE activation in other resistance models as appropriate (months 28-32)

We did not propose to start these studies in year 1.

TASK 4: Explore XBP-1 expression in clinical samples

a. Complete predictive study (months 24-30)
b. Complete prognostic study (months 30-36)

We moved up our initial studies on clinical samples to more rapidly assess whether XBP-1 protein is detectable in breast cancers and to begin exploring its potential as a predictive (improve prediction of endocrine responsiveness) and/or prognostic factor. We first established the optimum design for tissue microarrays and then measured expression of XBP-1 and several other proteins we have implicated in acquired antiestrogen resistance (12).

Construct and Characterize Tissue Arrays. Cores of tissues were arrayed on glass slides and then probed for gene/protein expression by immunohistochemistry (IHC) (17,21,22). We measured ERα and PgR (antibodies from Novocastra) because these proteins have power in predicting endocrine responsiveness. Tissue arrays were made by Dr. Singh (coinvestigator) using cases of breast carcinoma, invasive ductal type, not otherwise specified (NOS), selected from archives at LCC. An array of 480 cores from 54 breast carcinomas was constructed. Standard indirect immunoperoxidase procedures were used for IHC. Kappa (κ) statistics (minimax κ; average κ) were used to determine the smallest number of interpretable tissues that yield a satisfactory tumor classification when compared with the entire section (18). The minimax and average κ values show that ≥4 tissues with ER, and ≥5 tissues with PgR, provide an excellent classification of tumors' ER and PgR expression status (23). "Sensitivity" (rate of correctly classified positive tumors) and "specificity" (rate of correctly classified negative tumors) also were estimated. Optimal κ was found with 4 interpretable tissues for ER and 5 interpretable tissues for PgR. With ≥ 4 interpretable tissues for ER and ≥ 5 for PR, high sensitivity was found (>90%) for both genes. Specificity was 78% for ER and 86% for PgR. Since some cores may not be histopathologically interpretable, 7 cores are now used for all samples. These data show **(a)** our ability to generate high quality tissue arrays; **(b)** that 7 cores from each tumor can represent the heterogeneity of the entire original section for at least 2 different proteins; and **(c)** that we can use tissue arrays for the R33 studies.

Inbitial Study of XBP-1 in Breast Cancer Specimens. Using the genes from our initial comparisons (12) and the optimized breast cancer tissue arrays (above), high throughput IHC analyses confirmed the known co-expression of ER and PgR (Table 1). We find coexpression of NFκB and XBP-1 (antibodies from Santa Cruz). Coexpression of survival/mitogenic activities might be expected and there is some evidence suggesting that XBP-1 may be induced by NFkB. While some correlations are of borderline significance, we find IRF-1n (nuclear interferon regulatory factor-1; antibody from Santa Cruz) inversely correlated, and IRF-1c (cytosolic interferon regulatory factor-1) positively correlated, with both NFκB and XBP-1. IRF-1 is a transcription factor with tumor suppressor activity (13,30). Hence, we might expect activated protein to be in the nucleus (IRF-1n) and inactive protein to be in the cytosol (IRF-1c) and an inverse expression between IRF-1n and survival factors such as NFκB and IRF-1.

These data, while from a small study in cases without clinical outcomes data, are broadly supportive of our central hypothesis and clearly demonstrate detectable XBP-1 expression in breast cancers from women. Furthermore, these observations address a major component of Task 4, show that a more focused study in cases with clinical outcomes data is feasible, and that a larger study may provide interesting new insights into XBP-1 as a possible prognostic and predictive factor.

Table 1: *Correlation of IRF-1, XBP-1, and NFκB expression from tissue microarrays. *Numbers are p-values.* (-) = *inverse correlation,* (+) = *direct correlation. IRF-1c = cytoplasmic staining; IRF-1n = nuclear staining; NS=not significant.*

	ERα	PgR	IRF-1c	IRF-1n	NFkB
PgR	0.001 (+)	-	-	-	-
ErbB2	NS	0.005 (+)	-	-	-
IRF-1c	0.079 (+)	NS	-	-	-
IRF-1n	NS	0.014 (+)	0.088 (-)	-	-
NFkB	NS	NS	0.002 (+)	0.034 (-)	-
XBP-1	NS	NS	0.001 (+)	0.082 (-)	0.018 (+)

REPORTABLE OUTCOMES

We have completed and published our preliminary data and completed a review on antiestroegn resistance. We also have several abstracts that show how we are planning to publish our other data from this study.

1. Gu, Z., Lee, Y.R., Skaar, T.C., Bouker, K.B., Welch, J.N., Lu, J., Liu, A., Davis, N., Wang, Y. & Clarke, R. "Association of interferon regulatory factor-1, nucleophosmin, nuclear factor-κB and cAMP response element binding with acquired resistance to Faslodex (ICI 182,780)." *Cancer Res,* 8: 1155-1166, 2002.

2. Clarke, R., Liu, M.C., Bouker, K.B., Gu, Z., Lee, R.Y., Zhu, Y., Skaar, T.C., Gomez, B., O'Brien, K., Wang, Y., Hilakivi-Clarke, L.A. "Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling." *Oncogene,* 22: 7316-7339, 2003.

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CONCLUSIONS

Our emerging data are consistent with a potentially important role for XBP-1 in breast cancer. We have successfully overexpressed XBP-1 in MCF-7 cells, and hope shortly to engineer XBP-1 overexpression in T47D cells (transfections are now in progress). The evidence that XBP-1 binds to ERα is clearly supportive of a functional role in ERα action and is consistent with a potential role for XBP-1 in antiestrogen resistance. We have also optimized the use of tissue microarrays and demonstrated the detectable presence of XBP-1 protein in breast tumors. Of particular interest is the preliminary observation that XBP-1 may be coexpressed with NFκB, which is believed to induce XBP-1 in some cell systems.

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Association of Interferon Regulatory Factor-1, Nucleophosmin, Nuclear Factor-κB, and Cyclic AMP Response Element Binding with Acquired Resistance to Faslodex (ICI 182,780)[1]

Zhiping Gu,[2] Richard Y. Lee, Todd C. Skaar,[3] Kerrie B. Bouker, James N. Welch, Jianping Lu, Aiyi Liu, Yuelin Zhu, Natalie Davis, Fabio Leonessa,[4] Nils Brünner,[5] Yue Wang, and Robert Clarke[6]

Vincent T. Lombardi Cancer Center and Department of Oncology, Georgetown University School of Medicine, Washington, DC 20007 [Z. G., R. Y. L., T. C. S., K. B. B., J. N. W., A. L., Y. Z., N. D., F. L., R. C.]; Department of Electrical Engineering and Computer Science, The Catholic University of America, Washington, DC 20064 [J. L., Y. W.]; and Finsen Laboratory, Copenhagen, Denmark [N. B.]

ABSTRACT

To identify genes associated with survival from antiestrogens, both serial analysis of gene expression and gene expression microarrays were used to explore the transcriptomes of antiestrogen-responsive (MCF7/LCC1) and -resistant variants (MCF7/LCC9) of the MCF-7 human breast cancer cell line. Structure of the gene microarray expression data was visualized at the top level using a novel algorithm that derives the first three principal components, fitted to the antiestrogen-resistant and -responsive gene expression data, from Fisher's information matrix. The differential regulation of several candidate genes was confirmed. Functional studies of the basal expression and endocrine regulation of transcriptional activation of implicated transcription factors were studied using promoter-reporter assays.

The putative tumor suppressor interferon regulatory factor-1 is down-regulated in resistant cells, whereas its nucleolar phosphoprotein inhibitor nucleophosmin is up-regulated. Resistant cells also up-regulate the transcriptional activation of cyclic AMP response element (CRE) binding and nuclear factor κB (NFκB) while down-regulating epidermal growth factor receptor protein expression. Inhibition of NFκB activity by ICI 182,780 is lost in resistant cells, but CRE activity is not regulated by ICI 182,780 in either responsive or resistant cells. Parthenolide, a potent and specific inhibitor of NFκB, inhibits the anchorage-dependent proliferation of antiestrogen-resistant but not antiestrogen-responsive cells. This observation implies a greater reliance on their increased NFκB signaling for proliferation in cells that have survived prolonged exposure to ICI 182,780.

These data from serial analysis of gene expression and gene microarray studies implicate changes in a novel signaling pathway, involving interferon regulatory factor-1, nucleophosmin, NFκB, and CRE binding in cell survival after antiestrogen exposure. Cells can up-regulate some estrogen-responsive genes while concurrently losing the ability of antiestrogens to regulate their expression. Signaling pathways that are not regulated by estrogens also can be up-regulated. Thus, some breast cancer cells may survive antiestrogen treatment by bypassing specific growth inhibitory signals induced by antagonist-occupied estrogen receptors.

INTRODUCTION

ERs[7] are nuclear transcription factors, their activities being affected by the nature of the ligand bound and the pattern of genes/proteins expressed within cells (cellular context; Ref. 1). Antiestrogens compete with endogenous estrogens for activation of ER, and induce both cell cycle arrest and apoptosis in responsive cells (2). Neither the genes regulated by antiestrogens that signal to apoptosis nor those genes that confer an acquired antiestrogen resistance have been identified. Nonetheless, antiestrogenic drugs are effective in both premenopausal and postmenopausal breast cancer patients, and in the metastatic and adjuvant settings (3). The most widely used antiestrogen in current clinical practice is the triphenylethylene TAM. Clinical experience with this drug likely now exceeds 10 million patient years. When patients with metastatic disease are selected for treatment based on the ER and PgR content of their tumors, responses are seen in up to 75% of tumors expressing both receptors (2). TAM also reduces the incidence of ER-positive breast cancers in high risk women (4).

Other antiestrogens have emerged recently, most notably the benzothiophene Raloxifene and the steroidal ICI 182,780 (Faslodex). Both drugs appear to have significant clinical activity and may have better toxicological profiles when compared with TAM (2). Faslodex has significant activity in TAM-resistant patients (5), consistent with data obtained previously with TAM-resistant human breast cancer cells selected *in vitro* (6).

Despite the utility of antiestrogens, most tumors that initially respond to these drugs will recur and require alternative systemic therapies (2). Unfortunately, the precise mechanisms that confer resistance remain unknown. Change to an antiestrogen-stimulated phenotype has been described in some animal models (6, 7). This phenotype may occur in up to 20% of breast cancer patients but a loss of responsiveness to antiestrogens may be the more common phenotype (2). The expression of mutant ER proteins and splice variants has been reported but the functional role of these in endocrine resistance remains unclear (2). Most tumors acquiring antiestrogen resistance do so while retaining expression of ER (8). Thus, whereas lack of ER expression is a major form of *de novo* antiestrogen resistance, other mechanisms must be active in most instances of acquired resistance (2). The persistent expression of ER in tumors with acquired resistance suggests that some cells expressing this phenotype may either require ER expression and/or reflect the altered expression of otherwise estrogen-regulated genes.

Because ER-mediated transcription is directly affected by antiestrogens, we initially hypothesized that antiestrogen resistance might include perturbations in the patterns of expression and/or regulation of

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[2] Present address: Celera Genomics, 45 West Gude Drive, Rockville, MD 20850.

[3] Present address: Indiana University, Department of Medicine, Indianapolis, IN 46202.

[4] Present address: Laboratory of Clinical Investigation, National Institute on Aging, NIH, 5600 Nathan Shock Drive, Baltimore, MD 21224.

[5] Present address: United States Patent and Trade Mark Office, Crystal Plaza 3, Washington, DC 20231.

[6] To whom requests for reprints should be addressed, at Room W405A Research Building, Vincent T. Lombardi Cancer Center, Georgetown University School of Medicine, 3970 Reservoir Road, NW, Washington, D.C. 20007. Phone: (202) 687-3755; Fax: (202) 687-7505; E-mail: clarker@georgetown.edu.

[7] The abbreviations used are: ER, estrogen receptor; CRE, cyclic AMP response element; CCS-IMEM, improved minimal essential medium supplemented with 5% charcoal calf stripped serum; EGF-R, epidermal growth factor receptor; IRF-1, interferon regulatory factor-1; NPM, nucleophosmin; PgR, progesterone receptor; SAGE, serial analysis of gene expression; TAM, Tamoxifen; XBP-1, X-box binding protein-1; FACS, fluorescence-activated cell sorting; NFκB, nuclear factor κB; EGR-1, early growth response factor-1; TNFα, tumor necrosis factor α.

a subset of all of the ER-regulated genes (1). To address this hypothesis, we first generated a novel series of human breast cancer variants from the MCF-7 human breast cancer cell line. These cells have different growth requirements for estrogen and exhibit differential sensitivities to TAM and ICI 182,780 (9–11). In this study, we focus on MCF7/LCC1 cells (estrogen-independent, TAM-responsive, and ICI 182,780 responsive) and MCF7/LCC9 cells (estrogen-independent, ICI 182,780 resistant, and TAM cross-resistant; Ref. 11). Because the cells exhibit comparable cell cycle profiles[8] and are both MCF-7 variants, we can exclude the altered expression of genes related solely to differences in both genetic background and cell cycle distribution. A direct comparison of these respective transcriptomes should identify genes associated with survival from long-term antiestrogen exposure.

Several techniques are now available to explore the transcriptomes of tumors and experimental models. However, the most effective approach remains a matter of debate (12). Studies in breast cancer have been limited, most simply attempting to identify the genes expressed in breast cancers. For example, a recent study by Perou *et al.* (13) explored data from excisional breast biopsies from 42 individuals. Gene clusters, identified by exploration of the data structure, include those associated with ER, HER-2, and IFN-induced genes. A similar cluster of IFN-regulated genes was identified in the breast cancer cell lines included in the NIH drug screening program (14). Studies comparing the gene expression profiles of specific breast cancer phenotypes include an examination of histologically different samples from a single breast cancer lesion (15) and a preliminary analysis of a TAM-stimulated xenograft model (16). None of these reports directly addressed either the function or potential role of the specific genes identified. We have used two different but complementary approaches, SAGE and gene expression microarrays. These approaches would not be expected to provide identical data because not all of the genes identified by SAGE are on the microarrays, some genes identified on the cDNA arrays may be confounded by cross-hybridization to homologous RNAs, and the ability to detect significant differences between the SAGE databases is affected by the relative abundance of the tags and the size of the databases. We approached both technologies as means to sample the transcriptomes of MCF7/LCC1 and MCF7/LCC9 cells, and to generate data that would allow us to begin testing our hypothesis implicating estrogen-regulated genes in antiestrogen resistance. We now show that cells can survive prolonged antiestrogen treatment by altering the expression, patterns of regulation, and functional activation of specific estrogen-regulated genes.

MATERIALS AND METHODS

Cell Lines. MCF7/LCC1 cells were derived from the estrogen-dependent MCF-7 human breast cancer cell line after selection for growth in ovariectomized nude mice (9, 17). MCF-7/LCC9 cells were obtained by an *in vitro* stepwise selection of the estrogen-independent but antiestrogen-responsive MCF7/LCC1 cells against the steroidal antiestrogen ICI 182,780 (Faslodex). MCF7/LCC9 cells are ICI 182,780 resistant and TAM cross-resistant, express ER and PgR, and exhibit an estrogen-independent but responsive phenotype (11). MCF7/LCC1 and MCF7/LCC9 cells were routinely passaged in Improved Minimal Essential Medium without phenol red (Biofluids, Bethesda, MD) supplemented with 5% CCS-IMEM. Serum was stripped of endogenous estrogens as described previously and is estimated to contain ≤10 fM estrogen (18). Vehicle for all of the hormone/antihormone treatments was ethanol (final concentration <0.1% v/v). All of the cell cultures were maintained at 37°C in a humidified 5% CO_2:95% air atmosphere and shown to be free of contamination with *Mycoplasma* species as determined by solution hybridization to *Mycoplasma*-specific, radiolabeled, RNase riboprobes (Gen-Probe Inc., San Diego, CA).

SAGE Analyses. SAGE was performed as described previously (19). Polyadenylic acid mRNA was harvested from cells using biotin labeled-oligodeoxythymidylic acid magnetic beads (Promega PolyATract System 1000 kit; Promega, Madison, WI) and treated with DNase I enzyme to remove any contaminating DNA. mRNA (5 μg) was converted to double-stranded cDNA using the Life Technologies, Inc. cDNA Synthesis kit (Life Technologies, Inc., Rockville, MD). Biotinylated cDNA was completely cleaved with Nia III and the 3′-end digested fragments extracted with magnetic streptavidin beads. The cDNA was evenly divided and ligated, one half to linker A and the other half to linker B (19). Cleavage of the cDNA by BsmF1 produced 11–13 bp oligo DNA tags with linkers, which were blunt-ended with T4 polymerase. Linkers A and B were ligated together to form ditags, which were then amplified by PCR using primers to linkers A and B. Ditags (22–26 bp) were gel purified and ligated into concatenated polytags. The polytags were purified and cloned into the Sph1-digested pZeor1 vector, which was transferred to competent TOP10F′ cells by electroporation. Positive clones were selected overnight at 37°C for growth on low-salt Luria-Bertani bacterial plates supplemented with Luria-Bertani-Zeocin (50 μg/ml) and isopropyl β-D-thiogalactopyranoside (1 mM). Colonies were screened for plasmids containing appropriate inserts by size fractionating PCR products, obtained using M13 forward and reverse primers, in agarose gels. PCR products containing concatamers of >600 bp were purified and sequenced.

Characteristics of the SAGE databases are shown in Table 1. We compared the MCF7/LCC1 and MCF7/LCC9 databases, using the SAGE version 1.00 software (kindly provided by Dr. K. W. Kinzler, Johns Hopkins University, Baltimore, MD), to identify putatively differentially expressed genes. Only a representative sample of these can be presented. The genes presented in Table 2 were primarily selected based on: (*a*) fold difference ≥2-fold; (*b*) that the Tags compared should represent ≤2 genes; and (*c*) that a Tag found in either the MCF7/LCC1 and/or MCF7/LCC9 SAGE libraries must represent ≥0.10% of the database. Evidence that a gene was already known to be expressed in breast cancers also was considered. None of these criteria were considered an absolute requirement for gene selection. Whereas 2-fold was selected as the cutoff, biologically critical events can be controlled by genes that exhibit a fold regulation as small as 50% (20). As described recently by Man *et al.* (21), χ^2 analyses were used to compare the proportions of specific tags in each database.

RNA Isolation, Generation of Probes, and Hybridization of Gene Microarrays. Each probe was generated from an independent cell culture, each culture being grown on a different day but using identical cell culture conditions. Six MCF7/LCC1 and five MCF7/LCC9 cell cultures were used. RNA was isolated from proliferating, subconfluent monolayers of each cell line using the TRIzol reagent (Life Technologies, Inc., Grand Island, NY). RNA quality was determined by standard spectroscopic and gel electrophoresis analyses.

Probes for the Clontech Atlas gene microarrays (Clontech, Palo Alto, CA) were made as described by the manufacturer. Briefly, 1 μg of Dnase-treated mRNA was primed with the Clontech cDNA Synthesis Primer mix. The product was reverse transcribed into radiolabeled cDNA with [γ-^{32}P]dATP (Amersham Life Science Inc., Arlington Heights, IL), and the reaction incubated at 50°C for 25 min and terminated by adding 0.1 M EDTA (pH 8.0). Radiolabeled cDNA was purified and eluted through a NucleoSpin Extraction Column (centrifuged at 14,000 rpm). The cDNA probe was denatured with 1

Table 1 *Characteristics of the SAGE libraries from MCF7/LCC1 and MCF7/LCC9 cells*

Characteristics of SAGE libraries		Tags[a]	Gene hits
Tags sequenced from MCF7/LCC1 cells	12,816[b]	5,783	1
Tags sequenced from MCF7/LCC9 cells	11,109[b]	1,170	2
Number of Tags identified	10,518	208	3
Number of known Tags[c]	7,221	38	4
Number of unknown Tags	3,297	10	5

[a] Number of Tags representing a corresponding number of gene hits, *e.g.*, 5,783 Tags are specific for single genes, whereas 208 Tags could identify up to 3 genes each.
[b] Number of Tags in each SAGE database.
[c] Includes expression sequence tags.

[8] R. Clarke, unpublished observations.

Table 2 *Differentially expressed genes identified in the MCF7/LCC1 and MCF7/LCC9 SAGE libraries*

Putative gene[a]	Unigene no.	MCF7/LCC1	MCF7/LCC9	Difference[b]	P[c]	Gene function
N-ras-related gene	Hs.260523	2	20	10-fold	<0.001	G-protein
Cathepsin D	Hs.343475	7	34	5-fold	<0.001	Protease involved in tumor invasion
XBP-1	Hs.149923	7	25	4-fold	<0.001	Transcription factor
Prefoldin 5	Hs.288856	6	21	4-fold	0.002	Chaperone for unfolded proteins
HSP-27	Hs.76067	23	55	2-fold	0.001	Stress response protein
Vit B-12-binding protein	Hs.2012	17	37	2-fold	0.002	Vitamin-binding protein
NPM	Hs.9614	10	14	1.5-fold	>0.05	Oncogenic nucleolar protein
L14	Hs.738	13	2	6-fold	0.021	Ribosomal protein
Death-associated protein-6	Hs.336916	11	2	6-fold	0.049	Apoptosis-associated protein
EF-γ	Hs.2186	22	6	4-fold	0.014	Translation elongation factor
Ferritin, heavy polypeptide-1	Hs.62954	54	16	3-fold	<0.001	Iron-binding protein

[a] The gene designations are considered putative, although the identity of most genes designated in this fashion have been shown to be correct. These genes include those Tags where: (*a*) the fold difference is ≥2-fold; (*b*) the Tag could represent ≤2 genes; and (*c*) represents 0.1% of either the MCF7/LCC1 and/or MCF7/LCC9 SAGE library.

[b] Predicted fold difference in gene expression between MCF7/LCC1 *vs.* MCF7/LCC9 cells.

[c] Obtained by χ^2 analyses; *P* estimated to 3 significant figures.

[d] NPM (not statistically significant) is shown because we know it to be both estrogen regulated and associated with TAM treatment in patients.

M NaOH and 10 mM EDTA, and incubated at 68°C for 20 min. c_0t-1 DNA and 1 M NaH_2PO_4 (pH 7.0) were added to the denatured probe, and incubated at 68°C for an additional 10 min.

Each Atlas Array (Clontech) was prehybridized with 5 ml of ExpressHyb buffer (Clontech) and 0.5 mg of denatured DNA from sheared salmon testes at 68°C for 30 min with continuous agitation. The cDNA probe, prepared as described above, was then added and allowed to hybridize overnight. The array was washed four times with 2× SSC containing 1% (w/v) SDS for 30 min at 68°C and once with 0.1× SSC containing 0.5% (w/v) SDS for 30 min at 68°C. One final wash was performed with 2× SSC for 5 min at room temperature. The Atlas Array was sealed in plastic and signals detected by phosphorimage analysis using a Molecular Dynamics Storm phosphorimager (Molecular Dynamics, Sunnyvale, CA). Each filter was used only once.

Measuring NPM and EGF-R Protein Levels. Established methods were used for performing and quantifying Western analyses of NPM (22, 23). Briefly, 10 μg of protein was loaded onto an SDS-PAGE gel and fractionated under reducing conditions [5% (v/v) β-mercaptoethanol]. To account for within-gel differences, samples were loaded in a random sequence onto each gel. Proteins were blotted onto nitrocellulose membrane and the blots probed with an anti-NPM monoclonal antibody (kindly provided by Dr. Pui-Kwong Chan, Baylor College of Medicine, Houston, TX; Ref. 24). After transfer to the membranes, equal protein loading was confirmed by staining the nitrocellulose with Ponceau S as is widely reported (22, 23, 25). Any material remaining in the gels were stained by Coomassie Blue. This approach provides an adequate and appropriate estimate for equivalence of protein loading (22, 23, 25). Immunoreactivity was visualized using a horseradish peroxidase-linked goat antimouse IgG and the enhanced chemiluminescence detection system (Amersham Life Science Inc.). Chemiluminescence was densitometrically measured using a Quantity One Scanning and Analysis System (pdi, Huntingdon, NY).

EGF-R is expressed at low levels in MCF-7 cells and cannot readily be detected/quantified by Western blot. Consequently, we measured immunofluorescently labeled EGF-R protein by FACS. For each cell line, EGF-R immunofluorescence was performed by rinsing 5×10^6 cells once in PBS and pelleting cells by centrifugation at 1000 rpm for 5 min at room temperature. Cell pellets were resuspended in 100 μl of an anti-EGF-R mouse monoclonal antibody that recognizes the extracellular domain of the receptor (EGF-R antibody-1; NeoMarkers, Lab Vision Corp., Fremont, CA; 200 μg/ml diluted 1:50 in PBS), and incubated at room temperature for 1 h. Cell pellets were then resuspended in 1:50 dilution of R-phycoerythrin-conjugated goat antimouse IgG-2a (CALTAG Laboratories, Burlingame, CA) and incubated in the dark for 30 min. After rinsing in PBS, cells were again pelleted, fixed by resuspending in 1% paraformaldehyde, and fluorescence measured by FACS. Control cells were treated either with secondary antibody alone or with no antibody. FACS was performed on a FACStar^{Plus} flow cytometer (Becton-Dickinson, Mountain View, CA) at 488 nm.

RNase Protection Analysis of IFN Regulatory Factor-1 mRNA Expression. Total RNA was isolated using the TRIzol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. The IRF-1 riboprobe was made by *in vitro* transcription of a 360-bp fragment of the IRF-1 cDNA. The 36B4 loading control riboprobe was similarly obtained from a 220-bp fragment of the 36B4 cDNA (17). Riboprobes were labeled by the addition of [^{32}P]UTP (Amersham Life Sciences Inc.) in the transcription buffer. To achieve bands for the two genes with similar intensities, the 36B4 riboprobe was made with a specific activity of ~20% that of the IRF-1 riboprobe. The RNase protection assays were performed as described previously (26). Briefly, total RNA (30 μg), the IRF-1 riboprobe, and the 36B4 riboprobe were hybridized overnight at 50°C. After digestion with RNase A, the protected fragments were size fractionated on 6% acrylamide Tris-borate EDTA-urea minigels (Novex, San Diego, CA). The gels were dried and the respective signals quantified by phosphorimager analysis (Molecular Dynamics).

Estimation of the Transcriptional Activation of CREs and NFκB. Two commercially available promoter-reporter assays were used to measure NFκB and CRE transcriptional activities. Experiments were performed as described by the manufacturer (Stratagene, La Jolla, CA). Briefly, firefly luciferase reporter constructs, under the control of the appropriate enhancer elements and *trans*-activator constructs, were provided in the PathDetect *in vivo* signal transduction pathway *cis*-reporting system (Stratagene). Cells were grown to 90% confluence in 5% CCS-IMEM medium and seeded at 8×10^4 cells into each well of 24-well tissue culture dishes. After incubation for 12–24 h, cells were transiently transfected with the appropriate plasmids using the Qiagen Superfect transfection reagent as described by the manufacturer (Qiagen, Valencia, CA). The ratio of plasmid to Superfect reagent was 250 ng:1 μl, with a transfection time of 2.5 h.

Estrogen (5 nM) and ICI 182,780 treatments (10 nM) were administered for 48 h after transfection in CCS-IMEM. Transfected cells were harvested and firefly luciferase activity measured using the Stratagene assay system. Activity is expressed in relative light units from a 20-μl sample as detected by luminometry. Each measurement is from duplicate samples, independent experiments being repeated on different days. Normalization of transfection efficiency was made to the *Renilla* luciferase reporter construct, under the control of the cytomegalovirus promoter (Promega). The *Renilla* luciferase assay was performed using the Promega Dual-luciferase reporter assay system.

Assessment of Growth Response to Parthenolide. MCF7/LCC1 and MCF7/LCC9 cells were plated in 96-well tissue culture plates and incubated for 24 h in 0.2 ml of 5% CCS-IMEM. Medium was removed and replaced with fresh 5% CCS-IMEM containing either vehicle (0.1% DMSO) or parthenolide (300 nM and 600 nM). Cells were refed every third day with the appropriate cell culture medium. Cell growth was determined on day 6, using a crystal violet assay where dye uptake is directly related to cell number (27). Cells were incubated for 30 min with crystal violet stain [0.5% (w/v) crystal violet in 25% (v/v) methanol] at 25°C. Unincorporated stain was removed with deionized water and the cells allowed to dry at room temperature. Incorporated dye was extracted into 0.1 ml of 0.1 M sodium citrate in 50% (v/v) ethanol for 10–15 min at room temperature. Absorbance was read at 570 nm using a Molecular Devices V_{max} kinetic microplate reader.

Statistical Analyses and Analysis of Gene Expression Microarray Data. *t* tests were used to compare control and experimental groups as appropriate for the RNase protection, Western blot, promoter-reporter, and cell proliferation assays. All of the tests were two-tailed, with statistical significance established at $P \leq 0.05$, unless stated otherwise.

For the gene array studies, background signal was estimated locally and

subtracted from the signal obtained from its target cDNA, producing the background-corrected data. These corrections were done using the algorithms in Pathways 4.0 (Research Genetics Inc., Huntsville, AL). Background-corrected data were normalized to account for differences in probe-specific activity, hybridization, and other variables among replicates (28). Normalization was accomplished using the mean value of all of the background-corrected signals on each array.

Different approaches have been used to analyze data from gene array studies. Some methods are simply based on fold-regulation (29), others are more statistically based (16, 30), and/or apply an informatics-based exploration of data structure (31, 32). The optimal approach remains a subject of considerable debate (30). As with most gene microarray studies, our data set is high in dimensionality (597 dimensions) but the number of replicates is limited by the resource-intensive nature of the technology. The relatively few replicates limits the applicability of normal mixture models and other analyses that can operate in high dimensional data space (33, 34) and often generates noisy data sets.

Previously, we have reported a hierarchical visualization algorithm that can reveal all of the major aspects of the multimodal data points, which concurrently exist in a high dimensional gene expression space (35, 36). Using this algorithm, our data can be projected from 597 dimensions to two or three dimensions (multidimensional scaling). This is accomplished by respectively deriving the first three principal components fitted to the antiestrogen responsive (MCF7/LCC1) and resistant (MCF7/LCC9) gene expression data (Fig. 1). Thus, we evaluate the data structure subsets visually and assess whether these contain differentially expressed genes that may contribute to the respective phenotypes.

Because we can visualize data structure, our next priority was to identify a simple, supervised approach for reducing the dimensionality of the data without affecting its structure. Thus, we applied geometric and simple descriptive statistical approaches to the normalized data before and after a logarithmic transformation of these data. As noted previously, the distribution of the expression data for each gene is unknown (30), and it is unclear whether these violate the normal distribution required for parametric analyses. Indeed, it seems likely that the distribution assumption required will be normal for some genes and not for others. Whereas most investigators analyze data transformed by a logarithmic function, those genes with values that appear normally distributed before transformation may no longer have this distribution once transformed.

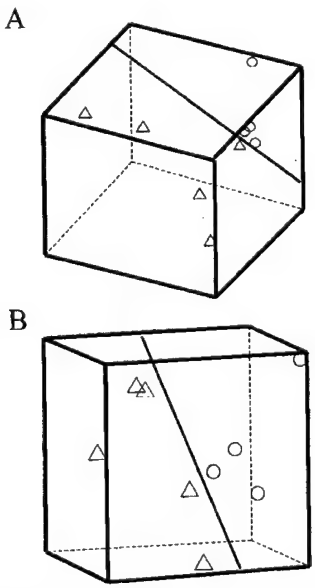

Fig. 1. Visual representations of the structure of the multidimensional gene microarray data. *A*, three-dimensional representation of 597 dimensions (△, MCF7/LCC1; ○, MCF7/LCC9) where the top three principal components capture 81.2% of the cumulative variance in the data. *B*, three-dimensional representation of 7 dimensions (data from Table 3) where the top three principal components capture 98.9% of the cumulative variance in the data. Axes represent the first three principal components derived from the gene expression data (79, 80). Plots are rotated to provide the optimal visualization. In both plots, a plane is shown demonstrating the linear separability of the MCF7/LCC1 ($n = 5$) and MCF7/LCC9 ($n = 4$) gene expression profiles.

To be inclusive, we used simple statistics (*t* tests) to explore the data. The inflated type-1 error from multiple comparisons should overestimate (false positive) significant differences. We considered this preferable to a high incidence of false-negative estimates, which would lead to the exclusion of potentially informative genes. The inclusion of uninformative genes (false negatives) is less problematic at this stage of the exploration. We used Student's *t* test, a *t* test for unequal variance (assumes normal distribution) and the nonparametric (distribution-free) Wilcoxon signed rank test. Logarithm transformed and nontransformed data were explored. This approach is similar to using a F test as described recently by Hedenfalk *et al.* (37).

t test results were evaluated and candidate genes selected with which to reconstruct a lower dimensional data set that should retain most of the information apparent in the top level visualization. However, the *t* test results were only one of several criteria used to guide gene selection, and only a subset of those genes that appear to be differentially regulated are presented. These genes were selected by comparing the results of *t* tests on logarithm transformed and untransformed data, fold-regulation (~2-fold or greater was selected because this difference is likely to be confirmed in independent analyses), the distribution of the background-corrected and normalized data for each gene (some genes appeared strongly differentially regulated but did not generate statistically significant differences because of heterogeneity in the data), and the probable relevance to breast cancer of each gene.

Where the gene subsets (reduced dimensional data) provide a reasonable description of the entire expression data, the replicate profiles of the resistant and responsive cells should exist in separable data space (35, 36). Furthermore, if the profiles are adequately defined by a small, rational gene subset, some of its members likely represent differentially expressed and functionally relevant genes. We acknowledge that our approach is limited, and is probably only applicable to simple comparisons within related cell culture models.

RESULTS

Genes Implicated by SAGE. The data in Table 1 show the number of different genes identified. Most genes were commonly expressed, and were not differentially expressed between the MCF7/LCC1 and MCF7/LCC9 cells. A selection of the genes identified by SAGE, and predicted to be differentially expressed in MCF7/LCC1 and MCF7/LCC9 SAGE databases, is shown in Table 2. Presentation of all of the genes expressed and/or differentially expressed is beyond the scope of a single, focused study.[9] The criteria applied for gene selection are described in "Materials and Methods." NPM was included because we already know it to be both estrogen regulated (23) and indirectly associated with TAM treatment in patients (38). Confirmation of the differential expression of NPM (see Table 2 and Fig. 2*B*) and altered CRE binding activity (the function of XBP-1; see Table 2 and Fig. 3*B*) indicate that these represent reasonable criteria for gene selection. Currently, the XBP-1 and NPM are the only genes from the SAGE database comparisons for which we have attempted to confirm differential expression/activation.

Comparing the SAGE databases identifies several genes that are up-regulated in MCF7/LCC9 cells compared with MCF7/LCC1 cells. These genes include *XBP-1, NPM, cathepsin D, HSP-27,* and n-*ras*. Increased CRE activity is indicated by the up-regulation of XBP-1, which regulates gene transcription through these response elements (39). XBP-1 is involved in regulating the expression of several tissue-specific genes including tissue inhibitor of metalloproteinases, osteopontin, and osteocalcin (40). Significantly, both Perou *et al.* (13) and West *et al.* (41) recently identified XBP-1 as being associated with ER gene expression clusters in human breast tumor biopsies. NPM is induced by estrogen in MCF-7 cells and is up-regulated in estrogen-independent cells (23). NPM also provokes an autoimmune

[9] http://clarkelabs.georgetown.edu/gu_et_al/gu_et_al_links.htm/.

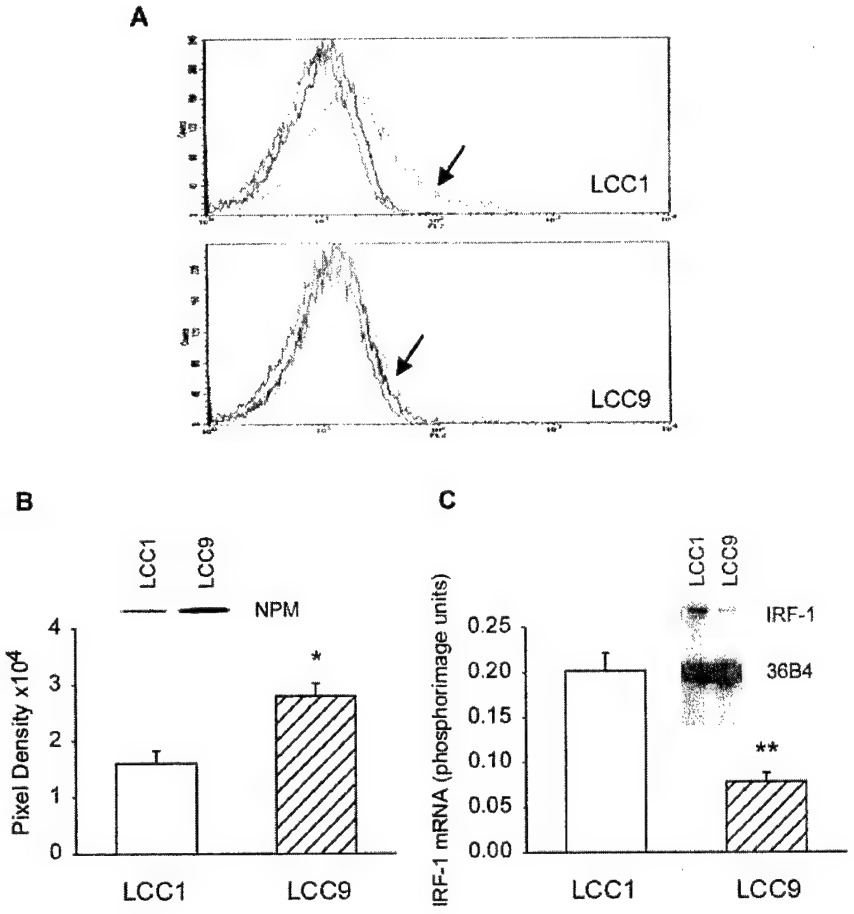

Fig. 2. Confirmation of the differential expression of NPM, EGF-R, and IRF-1 in MCF7/LCC1 and MCF7/LCC9 cells. *A*, EGF-R protein immunofluorescence as measured by FACS (representative figure of three experiments). *Arrows* indicate EGF-R signal, other signals are controls (no antibody; primary antibody but no secondary antibody). Axes are abscissa = fluorescence; ordinate = cell counts. *B*, NPM protein as measured by Western blotting ($*P \leq 0.02$) and represented as a percentage of control (MCF-7 cells growing in CCS-IMEM); *bars*, ±SE. Insert = representative Western blot. *C*, IRF-1 mRNA as measured by RNase protection ($**P = 0.005$, three independent replicate experiments) and expressed in phosphorimager units; *bars*, ±SE. Insert = representative analysis; 36B4 is a ribosomal gene (loading control).

response in breast cancer patients, the magnitude of which is associated with TAM therapy (38).

The altered expression of cathepsin D is consistent with our data published previously, showing increased secretion of this protein in several of our hormone-independent MCF-7 variants (42). Cathepsin D expression in breast tumors also is associated, at least in some studies, with a poor prognosis (43). HSP-27 expression has been implicated in refining the diagnosis of suspicious fine-needle aspirates of breast tissues (44). Vitamin B12 binding proteins are expressed in breast tumors (45), and vitamin B12 deficiency is a likely risk factor for breast cancer (46). Altered expression of the n-*ras*-related gene is consistent with the elevated *ras* signaling reported in some breast cancer cell lines and tumors (47).

SAGE also identified genes expressed at higher levels in the parental, antiestrogen-responsive cells (MCF7/LCC1) when compared with MCF7/LCC9 cells. These include ferritin, death-associated protein-6, and the eukaryotic elongation factor-γ. Ferritin is expressed in breast cancers, and breast tumor-derived ferritin may be a more useful tumor marker than measuring levels of ferritin in serum (48).

Structure of the Gene Microarray Data. It has been suggested that the cost required to perform gene microarray studies can be reduced by combining RNA populations from several replicates and performing a single hybridization on an Atlas array (16). However, we found heterogeneity among replicate experiments, which often remained after normalization. Logarithmic transformation of these data reduced this heterogeneity but not to the point where a single replicate could be used to obtain an adequate description of the data. Consequently, multiple replicates are required to provide a more reliable

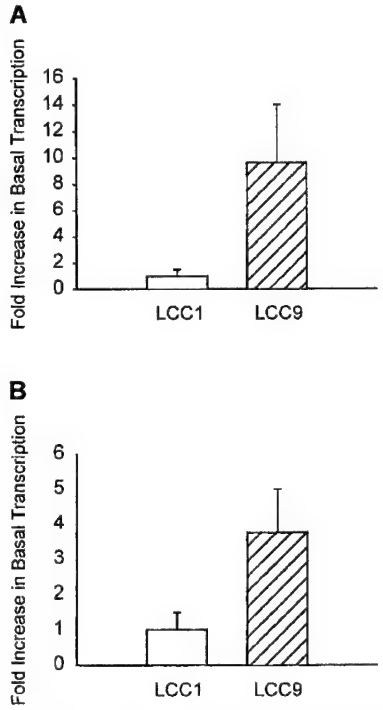

Fig. 3. Basal transcriptional activity of NFκB and CRE in MCF7/LCC1 and MCF7/LCC9 cells. *A*, NFκB. *B*, CRE. Data represent mean and are expressed as fold induction relative to MCF7/LCC1; *bars*, ±SE. All cells were grown in the absence of estrogens (CCS-IMEM).

Table 3 *Representative list of differentially expressed genes identified by gene microarray analyses*

Gene[a]	Unigene no.	MCF7/LCC1[b]	MCF7/LCC9	Gene function
NFκB	Hs.75569	1	2	Transcription factor involved in cell survival signaling
SOD	Hs.75428	1	2	Enzyme involved in detoxifying oxygen radicals
EGR-1	Hs.326035	3	1	Transcription factor
EGFR	Hs.77432	2	1	Growth factor receptor
IRF-1	Hs.80645	2	1	Transcription factor involved in signaling to cell cycle arrest and apoptosis
TNFα	Hs.241570	2	1	Cytokine
TNF-R1	Hs.159	2	1	Cytokine receptor involved in signaling to apoptosis

[a] Abbreviations are SOD, superoxide dismutase; TNF-R1, tumor necrosis factor-receptor 1.

[b] Data are represented as level of expression relative to the other cell line. Data are based on the mean values for each gene (6 microarrays of MCF7/LCC1; 5 microarrays of MCF7/LCC9). Values are expressed to the nearest integer.

estimate of the putative gene expression profiles. These observations on filter microarrays are consistent with recent reports for glass slide-based and oligonucleotide array-based gene expression microarrays (49, 50).

Fig. 1*A* is a visual representation of the multidimensional data (597 dimensions) in three dimensions. This visualization allows for an inspection of the data structure, and the likely comparability of the replicates among each other and between the two experimental groups (antiestrogen-responsive MCF7/LCC1 and antiestrogen-resistant MCF7/LCC9). For this top level visualization, the replicate gene expression profiles for MCF7/LCC1 and MCF7/LCC9 exist within linearly separable regions of the gene expression data space after elimination of one outlier array from each experimental group. The top three principal components capture 81.2% of the cumulative variance in the data (597 dimensions). Thus, the data structure is consistent with differences in the gene expression profiles as predicted by the known differential antiestrogen responsiveness of the two variants.

Genes Implicated by Gene Microarray Studies. The data in Table 3 show the fold-differences in expression of selected genes identified in the Clontech Atlas gene microarray studies selected using the criteria described in "Materials and Methods." The selection was not intended to describe fully the data set, only to assist in an initial exploration of the data. This small but rational subset of genes could be additionally evaluated in focused studies to confirm the differential expression patterns and establish potential functional relevance. Furthermore, if members of this subset were truly differentially expressed, we could begin to understand how cells perceive antiestrogens and adapt to this selective pressure.

To determine whether these genes are broadly representative of the differences between the gene expression profiles of MCF7/LCC1 and MCF7/LCC9 cells, we generated a three-dimensional projection from the seven-dimensional gene expression data space (Fig. 1*B*). This was necessary because we used several criteria to construct the subset, including some genes where fold-regulation or distribution of the data were given more weight than formal statistical significance. Consequently, we could not assume that we had maintained the linear separability of the data, at the top level, as seen in all 597 dimensions.

We might not expect this small subset of expression data (<2% of the information) to prove as effective in representing the respective phenotypes as the full data set (597 genes). Nonetheless, as for the 597-dimension visualization, after elimination of outlier data the seven-dimensional MCF7/LCC1 and MCF7/LCC9 profiles remain in linearly separable, three-dimensional data space. The top three principal components capture 98.9% of the cumulative variance in the data (seven-dimensions). This observation suggests that these data contain information that contributes to the differences in the molecular profiles of these two variants, that these genes may contribute to the respective biological phenotypes, and that additional studies of their potential functional relevance are warranted.

Genes expressed at a higher level in the MCF7/LCC1 cells include EGF-R, EGR-1, IRF-1, and both TNFα and its R1 receptor (TNF-R1). A well-established inverse relationship exists between the expression of EGF-R and ER in breast tumors (51). EGF-R can induce expression of EGR-1 (52), and expression of both genes is lower in MCF-7/LCC9 cells. EGR-1 is a transcription factor with proapoptotic activity (53) that can block NFκB function (54) and repress TGF-β receptor expression (29). EGR-1 expression is down-regulated in 7,12-dimethylbenz(*a*)anthracene-induced mammary adenocarcinomas in rats (55). IRF-1 is an IFN-regulated transcription factor that functions as a tumor suppressor gene (56, 57) and is induced by TNFα (58). A TNFα-mediated pathway for signaling to apoptosis occurs in MCF-7 human breast cancer cells (59, 60), and measuring serum TNF concentrations may be a useful prognostic marker in breast cancer patients (61). Furthermore, HER-2/*neu* can block resistance to TNFα-induced apoptosis in breast cancer cells, using a mechanism that involves activation of NFκB (62). We have previously implicated overexpression of superoxide dismutase in resistance to TNFα in MCF-7 cells (63). Superoxide dismutase appears to be up-regulated in MCF7/LCC9 cells (Table 3) and in TAM-stimulated MCF-7 xenografts (64). NFκB (p65/RelA) appears expressed at higher levels in MCF7/LCC9 cells. NFκB is overexpressed in ER-negative breast cancer cells (65) and has an important role in the development of the normal mammary gland (66).

NPM, EGF-R, and IRF-1 Are Differentially Expressed in MCF7/LCC1 and MCF7/LCC9 Cells. The data in Table 2 and Table 3 predict differential expression of NPM, EGF-R, and IRF-1 between MCF7/LCC1 and MCF7/LCC9 cells. To confirm these observations, we measured the levels of the EGF-R (immunofluorescence) and NPM proteins (Western blot) and IRF-1 mRNA (RNase protection). The data in Fig. 2*A* show that MFC7/LCC9 cells express lower amounts of EGF-R than MCF-7/LCC1 cells. NPM protein expression is significantly increased in MCF7/LCC9 cells compared with MCF7/LCC1 cells (Fig. 2*B*; $P < 0.02$), consistent with the predicted data from the SAGE analyses (Table 2) and our previous studies (23, 38). The higher levels of IRF-1 mRNA, seen in the antiestrogen-responsive MCF7/LCC1 cells in Table 3, are confirmed by RNase protection analysis (Fig. 2*C*; $P = 0.005$). Both the gene microarray and RNase protection analyses show an ~2-fold higher level of IRF-1 expression in MCF7/LCC1 cells, when compared with the antiestrogen-resistant MCF7/LCC9 cells.

Transcriptional Regulatory Activities of NFκB and CRE Are Increased in MCF7/LCC9 Cells. The increased expression of NFκB (gene expression microarray) and XBP-1 (SAGE) imply increased transcriptional activation of promoters containing NFκB and CRE response elements, respectively. We confirmed these observations directly, using commercially available promoter-reporter assays to measure transcriptional activities. The data in Fig. 3 show that the basal activity of both promoters is increased in MCF7/LCC9 cells; ~10-fold for NFκB and 4-fold for CRE ($P < 0.02$). The increase in transcriptional activation of the NFκB constructs is greater than that predicted by the gene array data, but mRNA, protein, and protein/DNA binding activities can be poor predictors of the functional activation of some transcription factors (67). This prediction is not problematic for XBP-1, where the 4-fold increase in mRNA expression identified by SAGE (Table 2) compares well with the 4-fold increase in basal transcriptional activation (Fig. 3*B*).

We next assessed whether ICI 182,780, the antiestrogen used to

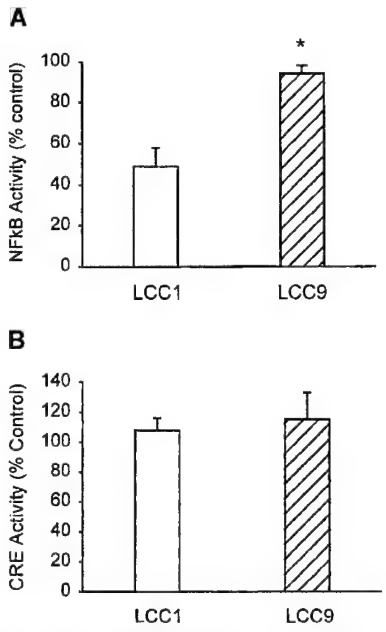

Fig. 4. Regulation of NFκB and CRE transcription by ICI 182,780 in MCF7/LCC1 and MCF7/LCC9 cells. *A*, NFκB (*$P \leq 0.001$, MCF7/LCC1 *versus* MCF7/LCC9). *B*, CRE (not significant). NFκB and CRE data are represented as mean of transcriptional activation expressed as a percentage of controls (vehicle-treated cells of the same cell line); *bars*, ±SE. Cells were grown in CCS-IMEM and treated with 10 nM ICI 182,780 for 48 h before measuring reporter gene expression.

generate the MCF7/LCC9 cells, could regulate the transcriptional activities of NFκB and CRE. Whereas ICI 182,780 inhibits NFκB activity in the MCF7/LCC1 cells (TAM- and ICI 182,780-responsive), this regulation is lost in the TAM and ICI 182,780 cross-resistant MCF7/LCC9 cells (Fig. 4*A*). In contrast, ICI 182,780 treatment does not alter the transcriptional regulatory activities of the CRE promoter in any of these variants (Fig. 4*B*).

MCF7/LCC9 Cells Are Specifically Responsive to an Inhibitor of NFκB Activity. The increased activation of NFκB and loss of its estrogenic regulation in MCF7/LCC9 cells suggests that these cells might now be partly dependent on NFκB signaling for survival/growth. Consequently, we compared the growth response of MCF7/LCC1 and MCF7/LCC9 cells to parthenolide, a potent and specific inhibitor of NFκB that can inhibit the inhibitor of NFκB kinase repressor of NFκB (68, 69) and also binds NFκB in a highly stereospecific manner to block DNA binding (70). Parthenolide produces a dose-dependent inhibition of MCF7/LCC9 cells, with an apparent IC_{50} of ~600 nM (Fig. 5). In contrast, parthenolide does not significantly affect growth of MCF7/LCC1 cells at these concentrations. MCF7/LCC9 cells are significantly more dependent on the transcriptional regulatory activities of NFκB than their ICI 182,780-responsive parental cells ($P < 0.01$ for MCF7/LCC9 *versus* MCF7/LCC1 at both 300 nM and 600 nM parthenolide).

DISCUSSION

We have begun to identify the molecular changes associated with cell survival after prolonged ICI 182,780 treatment in breast cancer cells. Whereas we have not attempted to confirm the altered expression of all implicated genes, some expression patterns are consistent with the activities we have confirmed. Here we discuss only those genes for which altered mRNA, protein, and/or transcriptional activation have been confirmed, and that are known to interact with each other in various cellular models, *i.e.*, IRF-1, NPM, NFκB, and CRE.

IRF-1 can function as a tumor suppressor and can signal to apoptosis through both p53-dependent and p53-independent pathways (71). These observations may partly reflect the ability of IRF-1 to induce a caspase cascade through activation of either caspase 1 (ICE; Ref. 72) and/or caspase 7 (73). Caspase 1 is involved in regulating apoptosis in normal mammary epithelial cells (74), and overexpression of caspase 1 is lethal in MCF-7 human breast cancer cells (75). Preliminary data from our laboratory demonstrate that overexpression of IRF-1 inhibits anchorage-dependent colony formation and that the rate of cell proliferation in MCF-7 cells is inversely related to the level of IRF-1 expression (76). These data suggest that the down-regulation of IRF-1 in MCF7/LCC9 cells may protect these cells from IRF-1-induced inhibition of proliferation and/or induction of apoptosis.

NPM can function as an oncogene, its overexpression fully transforming NIH 3T3 cells in a standard assay for oncogenic potential (77). We have shown that levels of autoantibodies to NPM increase in breast cancer patients 6 months before their recurrence. Consistent with an estrogenic/antiestrogenic regulation of NPM, the levels of these autoantibodies are lower in breast cancer patients that have received TAM (38). The increased NPM expression in MCF7/LCC9 cells compared with MCF7/LCC1 cells may reflect oncogenic potential of NPM, an activity potentially related to its ability to inhibit IRF-1 function (see below).

NFκB has been implicated in resistance to cytotoxic drugs and can function as a survival factor in various cell types (78). Several aspects of normal mammary gland development appear dependent on NFκB activity (66), perhaps partly reflecting its estrogenic regulation (65). Elevated NFκB activity arises early during neoplastic transformation in the rat mammary gland (79). Widely expressed in breast cancer cells and tumors, elevated NFκB activity is associated with estrogen-independence (65, 66). Currently, NFκB is the only protein known to induce BRCA2 expression (80). ICI 182,780 cannot suppress the increased NFκB activity in MCF7/LCC9 cells, despite inhibiting this function in ICI 182,780-responsive cells (MCF7/LCC1). The functional relevance of this observation was tested directly using parthenolide, which both specifically binds NFκB and blocks degradation of the endogenous NFκB inhibitor IκB, resulting in the inhibition of NFκB transcriptional regulatory activities (68, 70). This activity of parthenolide has been used to evaluate the functional role of NFκB in several recent studies (68, 69, 81, 82). MCF7/LCC9 cells are significantly more sensitive to growth inhibition by parthenolide than their MCF7/LCC1 parental cells. This

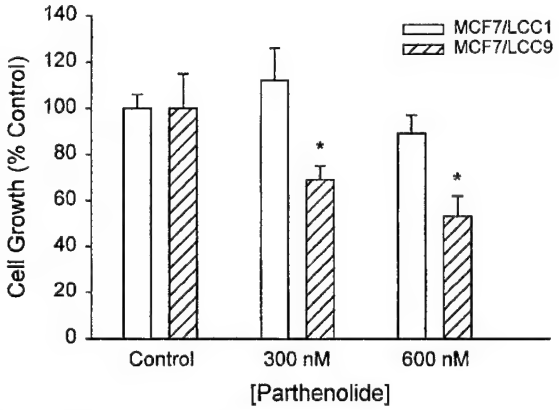

Fig. 5. Response to inhibition of NFκB activity by parthenolide. Data represent mean of four determinations, where absorbance in each treated population is expressed as a percentage of the absorbance in control cells (vehicle treated cells of the same cell line). *$P \leq 0.01$ MCF-7/LCC1 *versus* MCF7/LCC9. Cells were grown in CCS-IMEM without (control; vehicle only) or with parthenolide supplementation (300 nM; 600 nM).

observation is consistent with a greater functional reliance on NFκB activation for cell growth/survival, and implies that one option for surviving antiestrogen exposure is the up-regulation of an estrogen-regulated survival factor(s) concurrent with the loss of its ER-mediated regulation. Furthermore, parthenolide is now in clinical trials, and our data suggest that it may prove useful in combination with Faslodex or other antiestrogens to either increase responsiveness and/or delay the appearance of resistant disease.

XBP-1 has been identified recently in clusters of genes associated with ER-positive breast tumors in two independent studies (13, 41), and its expression is increased in MCF7/LCC9 cells. XBP-1 is a transcription factor that binds and activates CRE (39). The importance of CRE-regulated events is widely reported in many cell types (83, 84). These events include a likely role in signal transduction either at or downstream of ER and PgR (85). The relevance of increased CRE activity in MCF7/LCC9 cells is additionally supported by recent evidence that CRE-decoy oligonucleotides inhibit the growth of MCF-7 cells (86). We detected a 4-fold increase in CRE transcriptional activation in MCF7/LCC9 cells. Importantly, ICI 182,780 cannot regulate CRE activity in either MCF7/LCC1 (ICI 182,780-responsive) or MCF7/LCC9 (resistant) cells. These data imply an additional option available to breast cancer cells, a switch to signaling pathways that are normally independent of ER-mediated signaling.

IRF-1, NPM, NFκB, and CRE are known to affect cell proliferation, apoptosis, and/or carcinogenesis. Two critical protein-protein interactions directly link the IRF-1, NFκB, and NPM proteins. Direct binding occurs between IRF-1 and NPM (77), and between IRF-1 and NFκB (87, 88). In both cases, the interactions with IRF-1 have important effects on gene transcription and cell signaling. NPM binding inhibits the transcription regulatory activities of IRF-1 (77). A coordinated perturbation in the regulation of these two genes has occurred in the MCF7/LCC9 cells; NPM is up-regulated and IRF-1 is down-regulated. Thus, overexpression of NPM could additionally reduce the remaining lower levels of IRF-1, potentially blocking/eliminating its ability to initiate an apoptotic caspase cascade through caspase 1 and/or caspase 7. Such an effect would likely also eliminate the ability of IRF-1 to induce $p21^{cip1/waf1}$ (89) and cooperate with wild-type p53 in signaling to apoptosis (56, 57). Changes in the amount of available IRF-1 will directly affect the number of IRF-1:NFκB heterodimers available to regulate an additional series of genes. Whereas NFκB will compete with NPM for IRF-1 binding, their relative affinities for IRF-1 are unknown, and the preferred IRF-1 heterodimer remains to be established. IRF-1:NFκB protein-protein interactions or other cooperative interactions are implicated in the induction of ATF-2/jun (90), RANTES (91), VCAM-1 (88), interleukin 6 (92), and MHC class 1 genes (87). A functional IFN-β enhanceosome has been described that includes IRF-1, NFκB, and ATF2/jun (93). The importance of both IRF-1 and NFκB in IFN-induced signaling may contribute to the ability of IFNs to increase responses to antiestrogens (94–96).

CRE activation also may interact with the pathways regulated by IRF-1, NFκB, and NPM interactions. Delgado *et al.* (97) described a cyclic AMP-dependent pathway that inhibits IRF-1 transactivation. Thus, the increased CRE activity in MCF7/LCC9 cells may explain, in part, the lower IRF-1 mRNA levels seen both in the gene expression arrays and in the IRF-1 RNase protection studies.

The concurrent changes in NPM, IRF-1, NFκB, and CRE suggest a novel integrated signaling pathway that may involve the ability of NPM and CRE to inhibit IRF-1 initiation of a caspase cascade to apoptosis, the altered ability of cells to induce genes dependent on IRF-1:NFκB, and an increased activation of survival pathways that involve both NFκB and CRE. Studies to additionally establish the nature, function, and regulation of this putative pathway are currently in progress, including an overexpression of NFκB in sensitive cells and a dominant-negative approach in resistance cells. Because we looked only at cells that survived long-term antiestrogen exposure, the ability of the changes implicated in the present study to protect from an initial or short term exposure have yet to be determined. For example, cells may or may not survive an initial antiestrogenic exposure by the same mechanisms that allow for long-term survival. Irrespective of whether these other genes are functionally involved, their patterns of expression may be important in better predicting the 25% of ER+/PgR+, 55% of ER-/PgR+, and 66% of ER+/PgR− breast tumors that do not respond to antiestrogens (2).

It is not possible, in a single focused study, to define all of the potentially differentially expressed genes nor to establish their functional relevance firmly. Because the number of cellular models studied is small, additional functional studies where expression of the candidate genes is induced or repressed are in progress. Nonetheless, our data imply that breast cancer cells have highly plastic transcriptomes, with access to several signal transduction pathways for regulating the choice to differentiate, proliferate, or die. For example, MCF7/LCC9 cells have taken several possible interactive/interdependent approaches to circumvent the growth inhibitory effects of antiestrogens. This plasticity in gene expression patterns is consistent with the marked heterogeneity apparent in the clinical disease (2, 98).

In summary, our data suggest that one molecular profile associated with surviving prolonged antiestrogen exposure may include loss of ER-mediated signaling to apoptosis through IRF-1. This lost signaling is achieved both by down-regulation of IRF-1 and a coordinated up-regulation of its inhibitor NPM, and possibly another protein partner NFκB. Up-regulation of CRE activities also is implicated in this molecular profile. Other patterns of gene expression may provide alternative routes to the resistant phenotype or in cells that acquire a TAM-stimulated phenotype (2). The identification of these molecular profiles and signaling pathways may ultimately allow us to understand ER-regulated signaling, facilitate the development of novel treatment strategies, and allow clinicians to better identify antiestrogen-responsive and -resistant breast tumors.

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Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling

Robert Clarke*,[1], Minetta C Liu[1], Kerrie B Bouker[1], Zhiping Gu[2], Richard Y Lee[1], Yuelin Zhu[1], Todd C Skaar[3], Bianca Gomez[1], Kerry O'Brien[1], Yue Wang[4] and Leena A Hilakivi-Clarke[1]

[1]Department of Oncology and Vincent T. Lombardi Cancer Center, Georgetown University School of Medicine, 3970 Reservoir Rd NW, Washington, DC 20057, USA; [2]Celera Genomics, 45 West Gude Drive, Rockville, MD 20850, USA; [3]Indiana University Department of Medicine, Division of Clinical Pharmacology, Indianapolis, IN 46202, USA; [4]Department of Electrical Engineering and Computer Science, The Catholic University of America, Washington, DC 20064, USA

Antiestrogens include agents such as tamoxifen, toremifene, raloxifene, and fulvestrant. Currently, tamoxifen is the only drug approved for use in breast cancer chemoprevention, and it remains the treatment of choice for most women with hormone receptor positive, invasive breast carcinoma. While antiestrogens have been available since the early 1970s, we still do not fully understand their mechanisms of action and resistance. Essentially, two forms of antiestrogen resistance occur: *de novo* resistance and acquired resistance. Absence of estrogen receptor (ER) expression is the most common *de novo* resistance mechanism, whereas a complete loss of ER expression is not common in acquired resistance. Antiestrogen unresponsiveness appears to be the major acquired resistance phenotype, with a switch to an antiestrogen-stimulated growth being a minor phenotype. Since antiestrogens compete with estrogens for binding to ER, clinical response to antiestrogens may be affected by exogenous estrogenic exposures. Such exposures include estrogenic hormone replacement therapies and dietary and environmental exposures that directly or indirectly increase a tumor's estrogenic environment. Whether antiestrogen resistance can be conferred by a switch from predominantly ERα to ERβ expression remains unanswered, but predicting response to antiestrogen therapy requires only measurement of ERα expression. The role of altered receptor coactivator or corepressor expression in antiestrogen resistance also is unclear, and understanding their roles may be confounded by their ubiquitous expression and functional redundancy. We have proposed a gene network approach to exploring the mechanistic aspects of antiestrogen resistance. Using transcriptome and proteome analyses, we have begun to identify candidate genes that comprise one component of a larger, putative gene network. These candidate genes include NFκB, interferon regulatory factor-1, nucleophosmin, and the X-box binding protein-1. The network also may involve signaling through ras and MAPK, implicating crosstalk with growth factors and cytokines. Ultimately, signaling affects the expression/function of the proliferation and/or apoptotic machineries.
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Introduction

Antiestrogens primarily act by competing with estrogens for binding to the estrogen receptor (ER) and are the most widely administered endocrine agents for the management of ER-expressing breast cancers. The first antiestrogens were generated in the mid-1950s as fertility agents and included ethamoxytriphetol (MER-25) and clomiphene. The ability of these compounds to induce responses in some breast cancer patients soon became apparent (Kistner and Smith, 1960), but the compounds induced significant toxicity (Herbst *et al.*, 1964). In the early 1970s, the first study in breast cancer patients was published with a new antiestrogen tamoxifen (TAM, ICI 46474) (Cole *et al.*, 1971). Over the next 17 years, the total exposure to TAM reached 1.5 million patient years (Litherland and Jackson, 1988) and other selective estrogen receptor modulators (SERMs) are being developed and studied. TAM is now the most frequently prescribed antiestrogen, and compelling data have demonstrated a significant overall survival benefit with the administration of this agent in breast cancer patients with endocrine responsive disease (EBCTCG, 1992, 1998).

When compared with cytotoxic chemotherapy, antiestrogens are well tolerated and are associated with mostly minor toxicities (Love, 1989). Common side effects associated with TAM therapy include vasomotor symptoms, gastrointestinal disturbance, atrophic vaginitis, and changes in sexual functioning (Day *et al.*, 1999). While the frequency and severity of hot flashes and other toxicities can be particularly unpleasant for some women, remarkably few discontinue TAM because of these side effects. Medical indications for the

*Correspondence: R Clarke; E-mail: clarker@georgetown.edu

prompt discontinuation of therapy include associated venous thromboembolic disease and endometrial cancer (typically invasive adenocarcinoma, although uterine sarcomas have been reported). The incidence of these events is very low, and screening methods for both deep vein thrombosis and endometrial abnormalities exist. However, these increased risks must be considered in the light of the potential benefits—particularly in the case of healthy women considering TAM in the setting of chemoprevention as opposed to active treatment. The development of both venous thromboembolic disease and endometrial cancer is attributed to the estrogenic effects of TAM and may be abrogated by the development of more SERMs (e.g., raloxifene) or of pure ER antagonists (e.g., ICI 182,780; fulvestrant) (Robertson, 2001).

Some antiestrogens produce beneficial effects beyond their ability to inhibit existing breast cancers. The most convincing evidence supports an association between TAM treatment and a marked reduction in the risk of developing a contralateral breast cancer (EBCTCG, 1992) and a significant reduction in the incidence and severity of osteoporosis in postmenopausal women (Freedman *et al*., 2001; Kinsinger *et al*., 2002). Several early studies suggested a reduction in the risk of cardiovascular disease with TAM therapy, but this is not consistently reported (EBCTCG, 1998; Fisher *et al*., 1998). When observed, the cardiovascular benefit was usually attributed to the estrogenic effects of TAM; both estrogens and TAM produce apparently beneficial changes in serum triglyceride and cholesterol concentrations (Joensuu *et al*., 2000), perhaps through effects mediated by apolipoprotein E (Liberopoulos *et al*., 2002). However, these findings must be considered in the light of recent large studies of estrogenic hormone replacement therapy (HRT) that either failed to identify an HRT-induced reduction in coronary heart disease (Hulley *et al*., 1998; Grady *et al*., 2002; WHI, 2002) and stroke (Viscoli *et al*., 2001; WHI, 2002), or demonstrated an increase in the risk of these diseases.

An overview of antiestrogen resistance

Despite the relative safety and significant antineoplastic and chemopreventive activities of antiestrogens, most initially responsive breast tumors acquire resistance (Clarke *et al*., 2001b). It is unlikely that any single mechanism or single gene confers antiestrogen resistance. Rather, several mechanisms likely exist that encompass pharmacologic, immunological, and molecular events. These mechanisms, none of which are fully understood, likely vary within tumors. Intratumor variability in antiestrogen responsiveness will reflect the presence of multiple cell subpopulations (Clarke *et al*., 1990a). Since breast cancers appear highly plastic and adaptable to selective pressures, the intratumor diversity in antiestrogen responsive subpopulations also likely changes over time. Tumors appear capable of dynamically remodeling their cell populations in response to changes in host immunity or endocrinology, or the administration of local or systemic therapies. This plasticity is probably both cellular (some existing populations die out/back while other populations become dominant) and molecular (new cell populations emerge as individual cells/populations adapt their phenotypes by modifying their transcriptomes/proteomes).

Since the major pharmacologic and immunologic mechanisms of antiestrogen resistance have been previously reviewed (Clarke *et al*., 2001b), we will focus on the role of molecular signaling through ER-mediated activities in antiestrogen responsiveness. Antiestrogen resistance can be either *de novo* or acquired. The most common and best defined mechanism of *de novo* resistance is the absence of both ER and progesterone receptor (PR) expressions. However, we fail to predict response to antiestrogens in approximately 25% of ER+/PR+, 66% of ER+/PR−, and 55% of ER−/PR+ breast tumors (Honig, 1996). Many ER+ and/or PR+ breast tumors are already resistant by the time of diagnosis and the resistance mechanism in these tumors is unknown.

Overall, a loss of antiestrogen responsiveness by initially responsive tumors is likely to be the most common acquired resistance phenotype. Most initially antiestrogen responsive tumors retain levels of ER expression at recurrence on antiestrogen therapy that would still define them as being ER+ (Encarnacion *et al*., 1993; Kuukasjarvi *et al*., 1996; Bachleitner-Hofmann *et al*., 2002). Most data are for TAM treatment; ICI 182780, which causes degradation of ER (Dauvois *et al*., 1992), may have a greater potential for producing ER− tumors (Kuukasjarvi *et al*., 1996). From our *in vitro* studies, loss of ER is not required to achieve resistance to either ICI 182,780 or TAM (Brünner *et al*., 1993b, 1997). The loss of ER expression upon recurrence despite adjuvant TAM therapy has been reported in less than 25% of tumors (Kuukasjarvi *et al*., 1996; Bachleitner-Hofmann *et al*., 2002). Overall, a loss of ER expression does not seem to be the major mechanism driving acquired antiestrogen resistance.

A different resistance phenotype has been described in human breast cancer xenografts that exhibit a switch to a TAM-stimulated phenotype. This mechanism of clinical but not pharmacologic resistance may not be the dominant antiestrogen resistance phenotype. If the prevalence of acquired resistance phenotypes in ER+ tumors broadly reflects what is seen in *de novo* resistance, then the dominant resistance phenotype is a loss of antiestrogen responsiveness.

Whether the continued expression of ER is required for antiestrogen-resistant tumor growth or survival is not known. However, responses to aromatase inhibitors after an initial response and then failure on TAM are common (Buzdar and Howell, 2001) and strongly suggest that some TAM-resistant tumors retain a degree of estrogen responsiveness. Where durations of responses to second-line endocrine manipulations are short, truly estrogen-independent cell populations are either already present at the time of recurrence and/or many cells in the tumor are able to adapt rapidly to further changes in their endocrine environment. Very

short response durations or disease stabilization may reflect the withdrawal of a mitogenic stimulus that is not required for the survival or basal proliferation of most cells in the tumor.

Antiestrogens

TAM is a triphenylethylene and its triaryl structure has been widely copied in the design of new compounds. Several TAM derivatives are already available, including toremifene (chloro-tamoxifen) and droloxifene (3-hydroxytamoxifen). Not surprisingly, both drugs are essentially equivalent to TAM in terms of their antitumor activities and toxicities (Roos *et al.*, 1983; Pyrhonen *et al.*, 1999), so neither is widely used in clinical practice.

The characteristic of raloxifene that has attracted the most interest is its apparent lack of estrogenic effects in the uterus, resulting in great interest in this drug's potential role in breast cancer chemoprevention. Subgroup analysis of the data from the Multiple Outcomes of Raloxifene (MORE) trial revealed that administration of raloxifene was associated with a 75% reduction in the incidence of invasive breast cancer without a concurrent increase in the incidence of endometrial cancers (Cummings *et al.*, 1999). This finding has led to the ongoing randomized study of TAM and raloxifene (STAR) in breast cancer prevention. Raloxifene still acts as an antiestrogen in the brain, increasing the incidence of hot flashes (Davies *et al.*, 1999). A high incidence of severe hot flashes is problematic for a drug to be administered for approximately 5 years to otherwise apparently healthy women. Raloxifene was recently approved by the Food and Drug Administration for the treatment and prevention of osteoporosis in postmenopausal women. While a benzothiophene, raloxifene (keoxifene; LY 156,758) has a three-dimensional structure broadly similar to the triphenylethylenes.

ICI 182,780 (Faslodex; Fulvestrant) is among the more promising new antiestrogens. Unlike TAM, ICI 182,780 is a steroidal ER inhibitor that is often described as a 'pure' antagonist with no estrogenic activity. This is in comparison to the triphenylethylene and benzothiophene antiestrogens, which are nonsteroidal, competitive ER inhibitors with partial agonist activity. The pure antagonist is characterized by antineoplastic activity in breast cancer and is devoid of uterotropic effects. However, the lack of agonist activity limits beneficial effects in bone. Whether ICI 182,780 also will increase hot flashes depends on whether it reaches adequate concentrations in the brain. Unlike TAM (Clarke *et al.*, 1992), ICI 182,780 appears to be a substrate for the P-glycoprotein efflux pump (De Vincenzo *et al.*, 1996), a major contributor to the blood–brain barrier (Cordon-Cardo *et al.*, 1989). Consistent with this observation, initial studies suggest that this antiestrogen does not enter the brain in high concentrations (Howell *et al.*, 1996). Pure antagonists may further exacerbate bone loss, a concern that also applies to aromatase inhibitors (Dowsett, 1997), but this issue may be addressed with the concurrent use of bisphosphonates or other therapies for osteoporosis. Clinical experience with ICI 182,780 has been reviewed by Howell (2001).

Antiestrogens and breast cancer treatment

Antiestrogens are effective in the adjuvant, metastatic, and chemopreventive settings and clearly induce significant increases in overall survival in some breast cancer patients (EBCTCG, 1992, 1998). Unlike aromatase inhibitors (inhibit estradiol biosynthesis), which are administered as single agents only to women with nonfunctioning ovaries, TAM can be given irrespective of menopausal status. In the adjuvant setting, TAM is administered at a daily oral dose of 20 mg, and several studies have now shown that the optimal duration of treatment is 5 years. While shorter (2 years) and longer (10 years) treatment durations produce notable responses, the risk : benefit ratios are strongly in favor of 5 years of treatment (Stewart *et al.*, 1996; EBCTCG, 1998).

While molecular predictors of tumor responsiveness are rare for most breast cancer treatments, expressions of ER and PR strongly predict for a response to antiestrogens. Up to 75% of breast tumors expressing both receptors (ER+/PR+) respond to TAM. Response rates are somewhat lower in ER+/PR− tumors (~34%) and ER−/PR+ tumors (45%). The response rate in ER−/PR+ may be an overestimate; relatively few tumors with this phenotype have been evaluated and the ER− assessment may include false-negative ER measurements. Only a small proportion of ER−/PR− tumors respond to antiestrogens (<10%), perhaps also reflecting false-negative ER measurements. Indeed, the most recent meta-analysis from the Early Breast Cancer Trialists Collaborative Group (EBCTCG) found no significant reduction in recurrence rates in patients with ER-poor tumors who received adjuvant TAM (EBCTCG, 1998).

Results of the 1998 EBCTCG meta-analysis found limited evidence for a TAM-induced increase in the risk of death from any cause in women with ER-poor tumors. Why TAM might be detrimental to some women is unclear. However, ER− tumors are known to exhibit a more aggressive phenotype associated with lower rates of overall survival (Aamdal *et al.*, 1984) and would be expected to recur earlier and more frequently. Estrogenic effects of TAM in these women also could have increased the number of deaths from cardiovascular disease and stroke, reflecting the data noted above from recent studies of estrogenic HRT use (Viscoli *et al.*, 2001; WHI, 2002).

Antiestrogens and breast cancer chemoprevention

TAM's ability to inhibit contralateral breast cancers and relatively low incidence of serious side effects led to studies into its potential use as a chemopreventive agent for patients with a high breast cancer risk. Three large, randomized, chemoprevention studies with TAM have

been performed to date: the NSABP P-1 trial ($n=13\,388$ participants) (Fisher *et al.*, 1998), the Royal Marsden Trial ($n=2471$ participants) (Powles *et al.*, 1998), and the Italian Chemoprevention Trial ($n=5408$ participants) (Veronesi *et al.*, 1998). Outcomes have been mixed: no significant reduction in risk was seen in the initial reports of either the UK or Italian trials, whereas the P-1 trial reported significant reductions in the incidence of both noninvasive (50%) and invasive (49%) breast cancers. A recent update on the Italian Trial reports an 82% TAM-induced reduction in the breast cancer risk among women at high risk for ER+ breast cancer (Veronesi *et al.*, 2003). In the NSABP trial, reductions in breast tumor incidence were seen only in the incidences of ER+ tumors (Fisher *et al.*, 1998). Reasons for the disparities among the trials have been widely discussed; these tend to focus on differences in patient populations, subject eligibility criteria, and study size. Results from the NSABP P-1 trial, which are broadly consistent with the 39% reduction in contralateral breast cancer incidence reported for TAM use (EBCTCG, 1992), are usually considered the more definitive. These data contributed to the decision by the Federal Drug Administration (USA) in October 1998 to allow the use of TAM as a chemopreventive agent for breast cancer. More recently, NSABP has reported TAM-induced reductions in the risks of adenosis, fibrocystic disease, hyperplasia, metaplasia, fibroadenoma, and fibrosis in the P-1 trial (Tan-Chiu *et al.*, 2003).

Estrogens and breast cancer

Since antiestrogen action and resistance are intimately affected by estrogen exposure, we briefly address the role of estrogens in breast cancer. An association between parity and breast cancer risk was observed by the 16th century Italian physician Bernadino Ramazzini (1633–1714) in his '*De Morbis Artificium*' published in 1700. The ability of ovariectomy to induce remissions in premenopausal breast cancer patients was shown by the Scottish physician George Beatson, the first clear evidence of an effective endocrine therapy for this disease (Beatson, 1896). More recent epidemiologic data show clear associations of early age at menarche, late age at menopause (Nishizuka, 1992), pregnancy (Hsieh *et al.*, 1994), obesity (Hulka and Stark, 1995), serum estrogen concentrations (EHBCCG, 2002), and use of estrogenic HRTs (Magnusson *et al.*, 1999; Schairer *et al.*, 1999, 2000) or oral contraceptives (Berger *et al.*, 2000) with an increase in the risk of developing breast cancer. Risk appears related to the timing of exposure and whether the cancer develops during the premenopause or postmenopause (Hilakivi-Clarke *et al.*, 2002).

Precisely how estrogens affect breast cancer risk remains controversial and outcome may be dependent upon the timing and duration of exposure. During the postmenopausal years, estrogenic stimuli are more closely associated with an increased breast cancer risk. However, we have recently reviewed evidence consistent with the hypothesis that, depending on the timing of exposure, increased estrogenic exposure can be associated with a reduced risk of breast cancer (Hilakivi-Clarke *et al.*, 2002). For example, estrogenic stimuli during childhood or the premenopausal years may affect breast development such that the breast is less susceptible to transformation. Estrogens may reduce breast cancer incidence in some women by altering mammary gland development and inducing the expression of genes involved in DNA repair (Hilakivi-Clarke *et al.*, 1999a; Hilakivi-Clarke, 2000).

For the purposes of this review, we will focus on the aspects of estrogen exposure that are associated with increased breast cancer risk and the survival/proliferation of established neoplastic breast cells. Hence, estrogens can be considered to act either as promoters (factors that stimulate the growth and/or survival of existing transformed cells) or as initiators (factors that induce the genetic damage that leads to cellular transformation). Evidence that estrogens are tumor promoters is well established from both experimental and clinical observations. For example, the growth of several human breast cancer cell lines *in vitro* and *in vivo* is stimulated by estrogenic supplementation. Indeed, such estrogenic supplementation is effective whether administered as classical estrogens (e.g., estradiol, estrone, or estriol) or plant-derived phytoestrogens such as the isoflavone genistein (Hsieh *et al.*, 1998). In addition, antiestrogens, aromatase inhibitors, leutinizing hormone releasing hormone agonists/antagonists, and ovariectomy are effective in the treatment of some breast cancer patients, all of which limit the interaction between a promotional (estrogenic) stimulus and cancer cells.

As tumor promoters, the effects of estrogens are related to the duration and timing of exposure. Withdrawal of an estrogenic stimulus that acts as a promoter could produce an eventual reduction in risk because it no longer promotes the growth or survival of existing cancer cells. Pregnancy produces a natural and significant increase in circulating estrogens, but only a transitory increase in breast cancer risk in young women. Indeed, if the first pregnancy was at a young age, the short-term increase may eventually translate into a lifetime reduction in breast cancer risk (Hsieh *et al.*, 1994). The increased breast cancer risk associated with either oral contraceptive or estrogenic HRT use is also related to the recency of use. Risk begins to reduce with the cessation of use and is highest in current users (CGHFBC, 1996; Schairer *et al.*, 2000).

Evidence that estrogens act as chemical initiators is more controversial. Estrogens can exhibit carcinogenic activity in some animal models; perhaps the best-known example is the ability of estrogens to induce renal cancers in Syrian hamsters (Kirkman, 1972). However, compelling evidence that estrogens initiate mammary cancer in animals is hard to find. In the 1930s, Lacassagne (1932) performed several studies in male mice and showed that administration of large doses of estrone can induce mammary tumors. While consistent

with an estrogen-mediated initiation of mammary cancer, it is possible that the mice were infected with the mouse mammary tumor virus (MMTV). Other than some transgenic/null mouse models, only in the ACI rat does estrogen administration reproducibly produce a high incidence of mammary tumors (Cavalieri and Rogan, 2002).

Reactive estrogen semiquinone/quinone intermediates, produced by the redox cycling of estrogen metabolites hydroxylated at the C3 and C4 positions of the aromatic A-ring, are the most likely estrogen initiators (Cavalieri *et al*., 1997; Bishop and Tipping, 1998; Cavalieri and Rogan, 2002). These reactive species can generate a substantial intracellular oxidative stress and directly damage DNA through the production of DNA adducts. Such events could define reactive estrogen metabolites as initiators, rather than as merely promoters of carcinogenesis. Recently, the National Toxicology Program (2003) listed, for the first time, steroidal estrogens as carcinogens.

Estrogen independence and antiestrogen resistance

Estrogen independence and antiestrogen resistance are often considered to be synonymous, which is not surprising since ER− tumors are definitively estrogen-independent and very rarely respond to antiestrogens, ovariectomy, or aromatase inhibitors. Nonetheless, several observations suggest that various forms of both estrogen independence and antiestrogen resistance exist and that these may be biologically and clinically very different. For example, second-line responses to aromatase inhibitors after response and recurrence on TAM are common (Goss *et al*., 1995; Buzdar *et al*., 1996). Crossover between more similar compounds, such as other nonsteroidal antiestrogens, rarely produces secondary responses (Johnston, 2001), although crossover to structurally different antiestrogens can produce secondary responses in patients. Tumors that respond first to TAM (triphenylethylene) show a marked response to ICI 182,780 (steroidal) administered upon failure of the TAM therapy (Howell *et al*., 1995). Similar patterns of responses were seen previously in experimental models (Brünner *et al*., 1993b). For example, MCF-7 human breast cancer cells were selected for the ability to grow in the absence of estrogens (Clarke *et al*., 1989a). The selected cells are estrogen-independent because they no longer require estrogens for growth either in cell culture or as xenografts in athymic nude mice. However, when exposed to either 4-hydroxytamoxifen or ICI 182,780, the cells are growth inhibited both *in vitro* and *in vivo* (Clarke *et al*., 1989a; Brünner *et al*., 1993a, b).

These observations strongly imply that the ability of breast cancer cells to grow in a low or nonestrogenic environment is not always synonymous with antiestrogen resistance. Four antiestrogen resistance phenotypes have been defined (Clarke and Brünner, 1995) and are shown in Table 1. The clinical applicability of these phenotypes remains to be determined but they are useful for defining resistance phenotypes in experimental models.

Intratumor estrogens and antiestrogens and exogenous estrogenic exposures

Antiestrogens act within cells, primarily to compete with available estrogens for binding to ER. Thus, the antiestrogenic potency of any compound is related to its affinity for ER relative to that of any estrogens present and the concentrations of both the antiestrogens and estrogens. The data in Table 2 show the relative affinities of the primary estrogens, antiestrogens and their major metabolites, and selected environmental estrogens and phytoestrogens. Intratumor estrogen concentrations are affected by several factors including serum estrogen concentrations and local estrogen production within the breast. Serum estrogen concentrations are affected by the presence or absence of functional ovaries and exogenous estrogen use such as HRT, some oral contraceptives, and various dietary components.

Passive diffusion into cells across the plasma membrane appears to be TAM's and estradiols's primary method of entry into cells. However, both TAM and estrogens are extensively bound to serum proteins and probably also to cellular proteins in tumor/nontumor cells within the breast (Clarke *et al*., 2001b). Release from serum proteins likely occurs within the tumor vasculature, with both estrogens and antiestrogens being subsequently sequestered within tumor/nontumor cells by intracellular proteins. The lipophilicity of both hormone and drug, and the significant amount of adipose tissue in the breast, may produce a local reservoir for both estrogens and antiestrogens. However, the concentration of free drug/hormone within cells and serum may be relatively low. Intracellular sequestration of drug/hormone in tumor and stromal cells could produce a concentration gradient favoring

Table 1 Antiestrogen resistance phenotypes

Antiestrogen resistance	*Phenotype*
Type 1	Fully responsive to antiestrogens and aromatase inhibitors
Type 2	Resistant[a] to nonsteroidal antiestrogens but responsive to ICI 182,780 and aromatase inhibitors (or resistant to ICI 182,780 but responsive to nonsteroidal antiestrogens and aromatase inhibitors)
Type 3	Resistant to all antiestrogens but potentially responsive to aromatase inhibitors
Type 4	Multihormone-resistant (resistant to all endocrine therapies and includes ER− and PR− tumors)

[a]Resistance can be considered as unresponsiveness and antiestrogen-stimulated phenotypes

Table 2 Relative binding affinities (approximate) of selected estrogens, antiestrogens, and environmental estrogens and phytoestrogens[a]

Compound	*Relative binding affinity (17β-estradiol = 100)*	
	ERα	*ERβ*
Estrogens		
Estrone	60	37
Estriol	14	21
Antiestrogens		
Tamoxifen	7	6
4-Hydroxytamoxifen	178	339
Nafoxidine	44	16
ICI 164,384[b]	85	166
Raloxifene	69	16
Clomiphene	25	12
Environmental estrogens and phytoestrogens		
Genistein	5	36
Resveratrol	$<1.1 \times 10^{-4}$	$<1.6 \times 10^{-4}$
Zearalenol	7	5
o,p'-DDE 2(2-chloro-phenyl)-2-(4-chlorophenyl)-1,1dichloroethylene	<0.01	<0.01
Bisphenol A	0.01	0.01

[a]Adapted from Kuiper *et al.* (1998), Kuiper *et al.* (1997) and Bowers *et al.* (2000); the methods for estimating ER binding are not the same across these studies but all three express binding relative to the values estimated for 17β-estradiol. [b]ICI 182,780 is an analog of ICI 164,384

diffusion into local tissues. If the affinity and capacity of tissue for drug/hormone exceed that of blood, significant accumulation within tumors would likely occur. Data in Table 3 (adapted from Clarke *et al.*, 2001b) illustrate several points regarding the pharmacokinetics of estrogens and antiestrogens. For example, intratumor concentrations of both estradiol and TAM are much higher than their respective concentrations in the serum. For estrogens, where the primary estrogen present in tumors is 17β-estradiol, both biosynthesis within the tumor and significant uptake from blood occur.

The ability of estrogens and antiestrogens to compete for binding to ER is likely to reflect intracellular availability. While their respective free concentrations are largely unknown, the data in Tables 2 and 3 imply that many breast tumors should accumulate a sufficient excess of TAM and its major antiestrogenic metabolites to compete readily with intratumor estrogens. If the estimate for estradiol concentrations (1.29 nM) and the reported concentrations for TAM and its major metabolites ($\sim 3\,\mu$M TAM + $\sim 7\,\mu$M *N*-desmethyltamoxifen + $\sim 0.2\,\mu$M 4-hydroxytamoxifen) in tumors are good approximations (Table 3), antiestrogenic metabolites may accumulate to levels up to 10^4-fold higher than estradiol. While TAM and *N*-desmethyltamoxifen have relative ER binding affinities about 10% that of estradiol (Table 2), overall, antiestrogenicity may exceed estrogenicity in most TAM-treated breast tumors by 100-fold (assuming equivalent availability).

This interpretation is consistent with the initial antiestrogenic activity of TAM seen in most ER+ breast cancers. No compelling evidence shows that TAM becomes extensively metabolized to purely estrogenic metabolites in patients with antiestrogen-resistant cancer. Furthermore, little evidence has been produced to suggest that the balance of TAM metabolism is such

Table 3 Serum and intratumor estrogen and tamoxifen concentrations[a]

Serum concentrations		
Mean estimates of estrogen concentrations		*Comments*
Follicular phase	Luteal phase	
⩽0.28 nM	⩽1.1 nM	Normal menstrual cycle
Pregnancy		
⩽150 nM		Normal third trimester (when estrogen concentrations are highest)
Breast cancer	Controls	
0.114 nM	0.093 nM	All postmenopausal women; in most studies these differences are statistically significant[1]
Estimates of tamoxifen concentrations		
Concentration	Drug/metabolite	
⩽1.1 μM	Tamoxifen + metabolites	Similar to normal tamoxifen regimen
⩽4.0 μM	Tamoxifen	High-dose tamoxifen regimen
⩽6.0 μM	*N*-desmethyltamoxifen	High-dose tamoxifen regimen
Intratumor concentrations		
Mean estimates of estrogen concentrations		*Comments*
Breast tumors	Non-neoplastic	
1.29 nM	0.76 nM	Non-neoplastic includes adjacent normal, fibroadenomas, adipose tissues
Mean estimates of tamoxifen concentrations		
Concentration	Drug/metabolite	Mean estimates vary across studies. The values represented here are among the higher of the reported mean values[a]
⩽3.0 μM	Tamoxifen	Breast tumors
⩽4.0 μM	Tamoxifen	Brain metastases from breast cancer
⩽7.0 μM	*N*-desmethyltamoxifen	Breast tumors
⩽8.0 μM	*N*-desmethyltamoxifen	Brain metastases from breast cancer
⩽0.2 μM	4-Hydroxytamoxifen	Brain metastases from breast cancer

[a]See Clarke *et al.* (2001) as to how these values were obtained and for citations to the source publications

that sufficient concentrations of its estrogenic metabolites are produced to overcome TAM's intracellular cumulative antiestrogenicity (combination of parent drug plus its antiestrogenic metabolites) (Clarke *et al.*, 2001b). Currently, no clinically relevant ER variants/mutants have been described that could adequately affect intratumor pharmacology to an extent sufficient to offset this balance in favor of a TAM-stimulated or other antiestrogen-resistant phenotype in a significant proportion of breast cancers.

Changes in TAM influx/efflux could alter its intracellular concentrations, and limited evidence suggests that this may occur in some tumors. However, the extent to which it occurs and the mechanisms driving such changes are unclear (Clarke *et al.*, 2001b).

Exogenous estrogenic exposures and their effects on antiestrogen resistance

Since estrogens compete with antiestrogens for ER binding, any compound with either estrogenic activity or the ability to increase estrogen exposure could affect response to antiestrogens. Estrogenic exposures come in many forms, including plant and environmental estrogens (Hilakivi-Clarke *et al.*, 1999b; Clarke *et al.*, 2001a), dietary exposures that affect the levels of endogenous estrogens (Hilakivi-Clarke *et al.*, 1997), and estrogenic HRT (Clarke *et al.*, 2001b). Dietary antioxidant exposure also may affect antiestrogen responsiveness (Clarke *et al.*, 2001b) and some women already take the most potent natural antioxidant (vitamin E) as an alternative medicine for controlling menopausal symptoms (Stampfer *et al.*, 1993; Barton *et al.*, 1998; Koh *et al.*, 1999).

The inclusion of women on HRT in some of the chemoprevention trials has been one of the issues raised to explain the lack of TAM's activity in these trials. It is unlikely that HRT would raise serum estrogens beyond levels seen in TAM responsive premenopausal women. However, the nature of the estrogenic exposure is very different between postmenopausal women on HRT and premenopausal women. More data are required to assess directly the contribution of HRT to TAM responsiveness.

Dietary exposures and tamoxifen activity

Several dietary components, including those present in dietary fats, soy, fruits, vegetables, and alcohol, have been suggested to have either protective or harmful effects on the breast. Some of these dietary factors, such as dietary fats and soy, can alter circulating estrogen levels (Lu *et al.*, 2000) and interact with ER (Wang *et al.*, 1996b; Collins *et al.*, 1997; Zava and Duwe, 1997). TAM's ability to affect the growth of ER + tumor cells may be altered by dietary intakes of fats and soy. Fats, soy, and other dietary components also modify other cell signaling pathways (Agarwal, 2000; Bouker and Hilakivi-Clarke, 2000; Clarke *et al.*, 2002). If TAM signals through the same pathways, a dietary factor might modify TAM's ability to inhibit the growth of malignant breast cells (ER-dependent or -independent interactions). Dietary components that alter signaling of a pathway that affects tumor growth independent of TAM also could either potentiate or reverse TAM's effects. Data from both *in vitro* and *in vivo* studies strongly support the hypothesis that at least some dietary factors modify TAM's actions in the breast.

Soy, dietary fat, vegetables, and antiestrogen responsiveness

High soy protein intake has been proposed to contribute to low breast cancer incidence among Asian women (Adlercreutz, 1995). A recent meta-analysis shows that a high intake of soy is associated with a reduced risk of developing premenopausal, but not postmenopausal, breast cancer (Trock *et al.*, 2001). Soybeans contain large amounts of the isoflavones daidzein and genistein (Barnes *et al.*, 1994; Adlercreutz, 1995). Genistein has many biological effects that could potentially reduce breast cancer risk, including inhibition of tyrosine kinase, EGFR tyrosine phosphorylation, and topoisomerase II activities. It also arrests cell cycle progression at G_2–M, induces apoptosis, has antioxidant properties, modifies eicosanoid metabolism, and inhibits *in vitro* angiogenesis (see the review by Messina *et al.*, 1994). While each of these actions of genistein could influence antiestrogen responsiveness, they occur primarily at pharmacologic rather than physiologic exposures. Humans consuming high levels of soy-based food products have less than 1 μM of circulating genistein (Messina *et al.*, 1994), and 30–185 μM genistein is required to induce many of the above-mentioned effects in experimental models *in vitro* where bioavailability is already likely to be greater than *in vivo*.

At physiological concentrations, genistein exhibits estrogenic properties that could enhance breast cancer risk. Genistein activates the ER (Wang *et al.*, 1996b; Collins *et al.*, 1997; Zava and Duwe, 1997) and induces proliferation of human breast cancer cells *in vitro* (Martin *et al.*, 1978; Wang *et al.*, 1996b). Genistein also stimulates proliferation of mammary epithelial cells in rodents (Santell *et al.*, 1997; Hsieh *et al.*, 1998) and in women (Petrakis *et al.*, 1996; McMichael-Phillips *et al.*, 1998). Data from ovariectomized athymic mice, representing a model of postmenopausal breast cancer, show that genistein and soy protein isolate both promote the growth of MCF-7 xenografts (Allred *et al.*, 2001). Furthermore, a recent study in athymic mice showed that genistein blocked the inhibitory effect of TAM on the growth of MCF-7 xenograft (Ju *et al.*, 2002). These results suggest caution in consuming high levels of genistein among postmenopausal women who are taking TAM for their breast cancer or to reduce their risk of developing breast cancer.

Very little is known about possible interactions between high dietary fat intake and the activity of TAM. TAM has beneficial effects on some aspects of fatty acid metabolism, for example, by reducing cholesterol levels (Reckless *et al.*, 1997). Diets containing n-3 PUFAs can increase the efficacy of cytotoxic

drugs against ER− human breast cancer xenografts (MDA-MB-231) (Hardman *et al.*, 2001). A recent study suggests that n-3 PUFAs restore TAM's ability to inhibit cell growth (DeGraffenried *et al.*, 2003). Oleic acid appears to affect indirectly TAM's dissociation from cellular antiestrogen binding sites (Hwang, 1987), an effect that could increase the intracellular concentrations of free drug. Since n-3 PUFAs have many biological activities, they may play a role in modifying TAM's actions, including an ability to inhibit protein kinases (Mirnikjoo *et al.*, 2001). γ-linolenic acid has several properties that might make it antitumorigenic. Kenny *et al.* (2001) have shown that γ-linolenic acid reduces the growth of MCF-7 xenografts, reduces ER levels in these cells, and potentiates TAM's ability to inhibit cell growth. However, the precise mechanism of action of γ-linolenic acid remains to be determined.

Cruciferous vegetables, such as broccoli, cabbage, cauliflower, and brussel sprouts contain high levels of indole-3-carbinol (I3C) and its metabolite 3,3-diindolymethane (DIM). These compounds have been shown to exhibit chemopreventive activity in multiple target organs including the breast (Bradlow *et al.*, 1999). Several mechanisms of action have been proposed for I3C and DIM, including changes in phase I and II enzyme activities and in cell cycle progression. Data from Katchamart and Williams (2001) show that I3C and DIM downregulate the expression of the cytochrome *P*-450 components that convert TAM to its more potent metabolites. Thus, these authors propose that high intake of cruciferous vegetables might reduce TAM efficacy. Vitamin A/retinoids can interact with estrogens, and some studies suggest that retinoids can increase the activity of TAM (McCormick and Moon, 1986; Anzano *et al.*, 1994). Little evidence from human studies exists to support directly this interaction. However, remarkably few studies have been undertaken in this area and additional data are clearly needed.

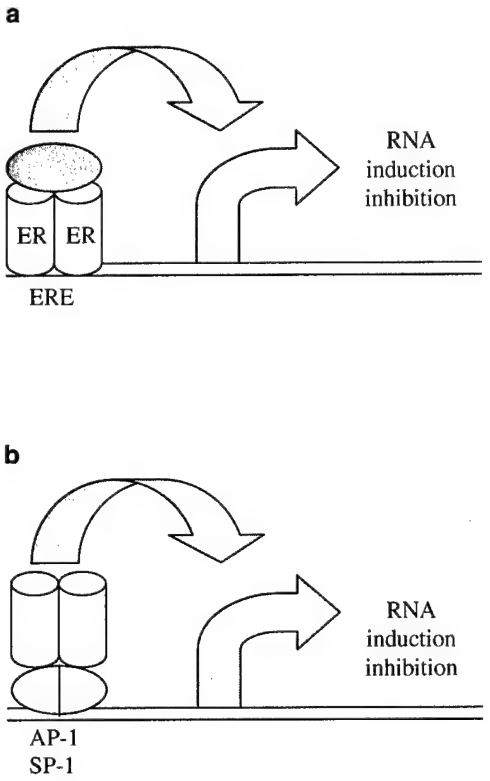

Figure 1 Estrogen receptor (ER) function—a simplistic representation. ERs function as nuclear transcription factors, bound to either estrogen responsive elements (**a**) or to proteins bound to other responsive elements, for example, AP-1, SP-1 (**b**). Transcription can be induced or repressed, with the pattern of genes affected likely reflecting the mix of coregulators available to bind to the various ER-transcription complexes formed on respective promoters. Evidence for both ligand-dependent and -independent activation exists, and it is clear that different ligands can induce different conformations in the bound ER proteins. ER = estrogen receptor; in (**a**) the hatched elipse represents a coregulator; in (**b**) the split elipse represents a protein complex such as AP-1 or SP-1

Estrogen receptors and antiestrogen resistance

Two ER genes have been identified: the classical ERα on human chromosome 6q25.1 and ERβ on chromosome 14q22–25. Each receptor acts as a nuclear transcription factor that binds responsive elements (estrogen responsive elements; EREs) within the promoters of target genes (Figure 1a) or binds to other proteins and affects their abilities to regulate transcription (e.g., AP-1, SP-1; Figure 1b). ERα and ERβ homology is limited in the transcriptional regulatory domains, particularly in the N-terminal region. Both ER homodimers and heterodimers are formed and these may differ in their ability to affect transcription at some promoters (Tyulmenkov *et al.*, 2000). For example, the ER binds directly to EREs, which are broadly defined consensus sequences with some tolerance to variation in their sequence. ER also binds to, and regulates the transcriptional activation of, other transcription factors including AP-1, SP-1, and at cyclic AMP response elements (CRE) (Paech *et al.*, 1997; Castro-Rivera *et al.*, 2001; Liu *et al.*, 2002b).

The patterns of ER expression vary in the mammary gland. In most normal mammary epithelia, the two receptors are rarely expressed in either a high proportion of cells or at very high levels. The ERα:ERβ ratio may change during carcinogenesis, such that the ERα proportion increases as the cells acquire a more progressed phenotype. Whether this change reflects an increase in ERα or a decrease in ERβ expression (Leygue *et al.*, 1998), and whether it is a function or a consequence of malignant transformation or progression is unclear. ERα appears to be the more highly expressed of the two receptors in breast tumors (Leygue *et al.*, 1998; Speirs *et al.*, 1999a), at least when both are coexpressed in the same cells (Saunders *et al.*, 2002). However, some of the few existing studies that measured both ERα and ERβ proteins have been complicated by the use of different antibodies of occasionally uncertain quality (Speirs, 2002).

When occupied by estradiol, ERα and ERβ can produce similar effects on gene regulation in simple

ERE-driven reporter construct studies (Kuiper *et al.*, 1996). However, the ligand binding profiles of the two receptors may be species specific (Harris *et al.*, 2002). Furthermore, at other promoters, the two receptors have very different activities. For example, ERα and ERβ have opposite effects on transcription driven by AP-1, SP-1, or CRE sites in promoter–reporter assays (Paech *et al.*, 1997; Castro-Rivera *et al.*, 2001; Maruyama *et al.*, 2001a; Liu *et al.*, 2002b). Differential regulation of cyclin D1 by ERα and ERβ has been reported (Liu *et al.*, 2002b), and ERβ can block the transcriptional activation of AP-1 by ERα (Maruyama *et al.*, 2001b). Changes in ER expression/activation might be important in affecting endocrine responsiveness if genes driven primarily by AP-1, SP-1, and/or CRE elements are rate limiting in affecting signaling to apoptosis/proliferation/survival.

The relative importance of ERα and ERβ in affecting antiestrogen responsiveness remains to be established. However, the extensive existing data with well characterized ERα antibodies that do not recognize ERβ allow for some speculation. Ligand binding ER assays (do not differentiate between ERα and ERβ) and immunohistochemical detection of ER in patients' tumors (detect ERα only) broadly agree in their determination of ER-positivity and prediction of TAM sensitivity (Alberts *et al.*, 1996; Molino *et al.*, 1997). Thus, whatever the role of ERβ, measuring ERα is sufficient to predict whether or not a patient is likely to benefit from treatment with antiestrogen, aromatase inhibitor, or ovariectomy. These findings also would be consistent with a requirement of ERα for antiestrogen sensitivity, which is further consistent with data from most experimental models in which ERα is usually the dominant ER isoform expressed.

Since loss of ERα (i.e., the tumor phenotype changes from ERα+ to ERα−) is relatively uncommon as an acquired antiestrogen resistance mechanism, it seems unlikely that many resistant tumors acquire a true ERα−/ERβ+ phenotype. If there is a role for ERβ, it may be driven by changes in its expression level relative to ERα, since heterodimers are functionally important (Pettersson *et al.*, 1997; Tyulmenkov *et al.*, 2000). When introduced into ER− MDA-MB-231 breast cancer cells, ERβ produces ligand-independent inhibition of proliferation, whereas ERα-mediated effects are ligand-dependent (Lazennec *et al.*, 2001). A ligand-independent suppression of growth by ERβ might confer a multihormone-resistant phenotype (Schinkel *et al.*, 1991) (multihormone resistance is Type 4 resistance as shown in Table 1), since ICI 164,384 could not block the ligand-independent effect of ER expression in MDA-MB-231 cells (Lazennec *et al.*, 2001).

Currently, determining the relative importance of ERβ expression in antiestrogen responsiveness is limited by the lack of adequate data regarding ERβ protein expression in responsive and resistant breast tumors. The possible association of ERβ mRNA expression with a poor prognosis (Dotzlaw *et al.*, 1999; Speirs *et al.*, 1999b) may further complicate matters. Only one small study ($n=9$ TAM resistant; $n=8$ TAM responsive tumors) has explored the association of ERβ expression with antiestrogen resistance. The authors reported increased ERβ mRNA expression in antiestrogen-resistant tumors (Speirs *et al.*, 1999a). Nonetheless, the outcome is potentially confounded by the very small number of cases, the fact that only ERβ mRNA was measured, and the possible association of ERβ expression with a more aggressive phenotype (Dotzlaw *et al.*, 1999; Speirs *et al.*, 1999b).

Several mutant and splice variant forms of both ERα and ERβ have been reported and previously reviewed (Hopp and Fuqua, 1998; Murphy *et al.*, 1998). Compelling evidence that any of these are functionally relevant in driving a significant proportion of breast cancers remains largely unconvincing. For example, most data only measure mutant mRNAs that may not be translated into biologically relevant protein concentrations in cells. Most tumors that express mutant ER concurrently express the wild-type receptor, with the mutant representing a relatively small proportion of total ER. A mutant ERα (D351Y) that perceives TAM as an agonist has been described in some TAM-stimulated MCF-7 cell variants (Jiang *et al.*, 1992). Similarly, changes in the F-region of the receptor also can affect the activities of estradiol and 4-hydroxytamoxifen (Schwartz *et al.*, 2002). The agonist activities of raloxifene are also increased in D351Y (Liu *et al.*, 2002a). Expression of this mutant in breast tumors in patients has not been reported. Thus, the clinical relevance of this ER mutant or functionally similar ER mutant proteins remains unclear. However, our understanding of the role of ER mutants and variants may change in the near future (Fuqua, 2001). Currently, little compelling evidence exists in support of mutant or splice variant ERα and/or ERβ contributions to either *de novo* or acquired antiestrogen resistance or hormone independence (Karnik *et al.*, 1994; LeClercq, 2002). However, the importance of receptor mutations and varinats in other diseases suggests that a role for these modifications of ERs may yet be shown to be important.

Coregulators of estrogen receptor function and antiestrogen resistance

Whatever the ERE and/or other transcription factor bound, the ability to affect transcription of a target gene is further modified by multiple components of the transcription complex. Perhaps the most widely studied modifiers of ER-mediated transcription are the coregulators. Coregulators can be either coactivators (inducers) or corepressors (inhibitors) of gene transcription. These molecules often act by altering histone acetylation (Kim *et al.*, 2001). While most studies of coregulator action have been carried out with ERα, ERβ function is also affected (Tremblay *et al.*, 1998), as is the activity of other members of the steroid hormone receptor superfamily.

ER coregulators in several protein families have been described in recent years, almost all of which are ubiquitously expressed (Graham *et al.*, 2000) and defined initially by their ability to affect ER-mediated

transcription in simple promoter–reporter transcription assays. Considerable redundancy is evident, with many coactivators or corepressors exhibiting similar transcription regulatory effects in comparable/identical biological assays. A full understanding of the role of coregulators may be further complicated by gene promoter-, tissue-, and species-specific effects, all of which contribute to the cellular context. Thus, the pattern of other proteins expressed in a cell (cellular context) may greatly influence how and whether a specific coregulator is the dominant effector in regulating a ligand's ability to affect ER-mediated transcription (Clarke and Brünner, 1996; Clarke *et al*., 2001b).

The ability of an ER-driven transcription complex to recruit coregulators can be strongly ligand-dependent. For example, 4-hydroxytamoxifen induces a conformation that blocks the coactivator recognition groove in ER (Shiau *et al*., 1999). Estrogens and antiestrogens have long been known to affect the physical properties of ERs (Miller *et al*., 1984). The importance of ligand to receptor conformation and activation led to early conceptual models that have received renewed attention in recent years. Perhaps the most important information has come from crystallographic studies of the ER binding domain complexed with different ligands (Brzozowski *et al*., 1997; Pike *et al*., 1999; Shiau *et al*., 2002). Several laboratories have used these data to describe conceptually similar models of ER function when liganded with either agonists or antagonists (Wurtz *et al*., 1998; Pike *et al*., 1999; Liu *et al*., 2002a, Shiau *et al*., 2002). The major limitations of such studies are the use of only the ligand binding domain (requires the assumption that no other domains of the ER affect its structure) and the use of crystal structures that may or may not fully reflect receptor structure in the more complex environment of a living cell. Nonetheless, data from such studies can provide important molecular insights into important biological responses.

The consequences of ligand-specific ER conformations are becoming evident but may be complex (McKenna *et al*., 1999). The coactivator SRC-1 produces a ligand-independent activation of ER while enhancing the agonist activity of the potent TAM metabolite 4-hydroxytamoxifen (Smith *et al*., 1997). SRC-1 also interacts synergistically with CRE binding proteins in regulating ER-mediated transcription (Smith *et al*., 1996). SMRT (corepressor) binds ER and blocks the agonist activity of 4-hydroxytamoxifen induced by SRC-1 (Smith *et al*., 1997). N-CoR is a corepressor that binds TAM-occupied but not ICI 182,780-occupied ER (Jackson *et al*., 1997). The functional relevance of this latter observation is consistent with the lack of full crossresistance between these two drugs in cell cultures models (Brünner *et al*., 1993b) and in breast cancer patients (Howell *et al*., 1995; Robertson, 2001). However, a recent study found no association between N-CoR expression and outcome in TAM-treated patients (Osborne *et al*., 2002).

It might be expected that increased expression or function of a protein that allows an antiestrogen to act as an agonist, or decreased expression of a coregulator that suppresses ER activity when the receptor is occupied by an antiestrogen, could confer a degree of antiestrogen resistance (Clarke and Brünner, 1996; Clarke *et al*., 2001b). Evidence for this in human cancers and experimental models remains somewhat limited. Expression of the corepressor N-CoR is lower in TAM-stimulated MCF-7 xenografts than in wildtype xenografts (Lavinsky *et al*., 1998), but the functional relevance of the observation in human cancers is unclear. Chan *et al*. (1999) studied a small cohort of TAM-resistant human breast tumors ($n = 19$) but found no difference in the expression of TIF-1, RIP140, or the corepressor SMRT. Lower levels of the coactivator SUG-1 were detected in some TAM-resistant tumors, but the consequences for antiestrogen responsiveness of reduced SUG-1 expression require further study.

Extrapolating many of these observations to specific biological functions in breast tumors is not always a simple matter. For example, most data have been obtained, of necessity, from the use of somewhat artificial experimental models with simple promoter conformations. ERE structure is variable across known estrogen-regulated genes, and a promoter's ability to bind ERs and coregulators can be affected by its local structure (Truss and Beato, 1993; Nardulli *et al*., 1995; Lee and Lee, 2001). Different ER–antiestrogen complexes also may recognize different promoter elements (Yang *et al*., 1996). Thus, promoter context is likely to be important (Clarke and Brünner, 1996). Given the evidence of considerable coregulator redundancy and ubiquitous expression (McKenna *et al*., 1999; Planas-Silva *et al*., 2001; McKenna and O'Malley, 2002), it is unclear whether measuring or affecting changes in the expression/function of any single coregulator will prove clinically useful. Attempting to affect resistance by modifying the expression of any single coregulator could be confounded by compensatory responses in other coregulators, as likely happens for mammary gland development in SRC-1 (Xu *et al*., 1998) and E6-AP null mice (Smith *et al*., 2002). A greater degree of specificity will likely be obtained by targeting specific genes within a functionally relevant gene network (Clarke and Brünner, 1996), which would be downstream of any coregulator activities. The overall balance in the patterns and levels of expression of coactivators and coregulators also likely contributes to ER signaling and endocrine responsiveness. Clearly, cellular context is critical in assessing the role of specific coregulators in affecting a given phenotype (Clarke and Brünner, 1996; Clarke *et al*., 2001b).

In summary, with such redundancy and apparent lack of cell/tissue specificity, measuring the expression of specific coregulators to predict an antiestrogen-resistant phenotype may be uninformative, and affecting changes in the expression/function of any single coregulator to alter phenotype may prove difficult. We still do not know with any certainty which estrogen-regulated genes are responsible for affecting cell proliferation, cell survival, or apoptosis in breast cancer. Hence, we do not know the structure of their promoters, the coregulators their occupied receptors can recruit into

functional or inactive transcription complexes, or the cellular context in which they exist in responsive and resistant cells.

Estogen receptor-independent cell signaling in antiestrogen resistance

Only a small proportion of ER−/PR− tumors respond to antiestrogens, consistent with their primary actions being mediated by ER. Nonetheless, many investigators have explored ER-independent signaling as mechanisms of antiestrogen resistance. The primary role of these effects is unclear and some occur at concentrations that are not pharmacologically relevant. Nonetheless, such activities can alter ER function or may interact with signaling downstream of ER (Figure 2). Since these mechanisms have been reviewed in detail (Clarke *et al.*, 2001b), we now only briefly discuss some of the more relevant.

Antiestrogen-induced induction of oxidative stress responses is perhaps the most widely studied ER-independent mechanism. The redox metabolism of several TAM metabolites can give rise to reactive species that can induce oxidative stress (Ye and Bodell, 1996), and both TAM and 4-hydroxytamoxifen produce 8-hydroxy-2′deoxyguanosine (Okubo *et al.*, 1998). TAM's ability to induce quinone reductase (Montano and Katzenellenbogen, 1997), protein kinase C redistribution (Gundimeda *et al.*, 1996), and lipid peroxidation (Schiff *et al.*, 2000), and our observations that antiestrogen-resistant cells upregulate cytochrome *c* oxidases (Gu *et al.*, 1997) and NFκB (Gu *et al.*, 2002) also are consistent with antiestrogen effects on oxidative stress responses (reviewed by Clarke *et al.*, 2001b).

Other ER-independent effects include perturbations in membrane structure (Clarke *et al.*, 1990b), changes in protein kinase C activation and subcellular localization (O'Brian *et al.*, 1986; Gundimeda *et al.*, 1996), and inhibition of the intracellular Ca^{++} binding protein calmodulin (Rowlands *et al.*, 1995). Some of these effects may be inter-related, since inhibition of protein kinase C also blocks calmodulin-dependent EGFR transactivation (Tebar *et al.*, 2002). These latter mechanisms may arise independent of ER, but would affect ER-mediated signaling. Calmodulin has been implicated as a coregulator of ER action (Biswas *et al.*, 1998), and EGFR-mediated signaling through MAPK may affect ER activation (see for recent reviews Clarke *et al.*, 2001b; Santen *et al.*, 2002).

The extent to which these mechanisms are truly ER-independent, in that they do not affect any aspect of ER-mediated signaling, requires further study. As with TAM's effects on calmodulin, ER-independent interactions may have significant effects on ER activation and function. For example, several growth factors appear to be able to activate ER through the induction of MAPK activities capable of changing ER's phosphorylation status (Clarke *et al.*, 2001b; Santen *et al.*, 2002). Other ER-independent events may interact with ER-mediated signaling downstream of ER activation. Despite these many activities, ER expression is required for most cells to respond to antiestrogens. While the importance of ER-independent signaling is unclear, many such signals may be necessary but not sufficient for affecting antiestrogen responsiveness (Clarke *et al.*, 2001b).

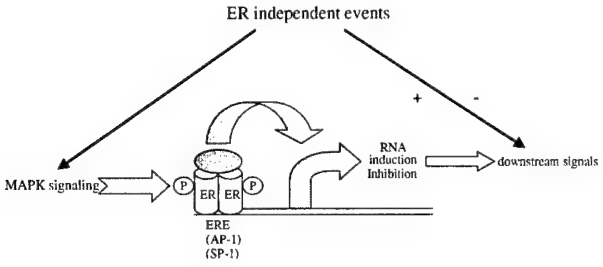

Figure 2 Putative role of estrogen receptor-independent effects of steroids and antiestrogens. These activities are induced by hormones or antihormones that are not directly mediated by their interactions with ERs. Such effects may be necessary, but they are not generally sufficient, to elicit a proliferative/antiproliferative response at most physiologically or pharmacologically relevant concentrations. ER-independent events may affect ER signaling either by altering ER activation and/or regulating the expression/function of other genes/proteins that are induced/repressed downstream of directly ER-regulated transcriptional events. The hatched elipse represents a coregulator; Ⓟ = phosphorylation

Antiestrogens, apoptosis, and cell death

Antiestrogenic exposures produce a G_0/G_1 cell cycle arrest (Taylor *et al.*, 1983), whereas estrogenic exposures are primarily mitogenic and increase the proportion of cells in S and G_2/M while reducing the proportion in G_0/G_1. Such effects are generally consistent with a cytostatic rather than cytotoxic effect. However, in our experience, long-term selection against antiestrogens *in vitro* or prolonged estrogen withdrawal from estrogen-dependent cells also induces cell death. Similar effects are seen in animal models. These observations are consistent with the ability of antiestrogens to reduce the incidence of ER+ breast cancers in high-risk women (chemoprevention) and produce an overall survival benefit in breast cancer patients (treatment). Initially, antiestrogens may produce a cytostatic effect that, in the longer term, results in cell death.

The precise mechanisms signaling to and responsible for antiestrogen-induced cell death are not fully understood. Most studies are consistent with an induction of an apoptotic or programmed cell death (Kyprianou *et al.*, 1991; Huovinen *et al.*, 1993; Zhang *et al.*, 1999). However, many breast cancers that acquire antiestrogen resistance still respond well to cytotoxic drugs, many of which also signal to apoptosis (Wang *et al.*, 1996a). Such effects could not occur if the machinery for inducing apoptosis was no longer intact or functional. Thus, the effects of antiestrogens must be upstream of effector mechanisms and reflect subtle changes in how ERs affect signaling to apoptosis. Other signaling pathways also may be important. Data from a recent study suggest

that adjacent normal mammary cells can induce cell death through Fas signaling in breast cancer cells. Resistance to this effect in some breast cancer cells was restored by inhibition of NFκB and PI3 kinase (Toillon *et al.*, 2002).

Tamoxifen-stimulated phenotype in antiestrogen resistance

While antiestrogens can induce growth arrest and apoptosis, in some patients, initiation of TAM therapy is associated with rapid progression of their disease, although continuation of TAM generally produces a beneficial response (Plotkin *et al.*, 1978; Clarysse, 1985). This response is called 'tumor flare' and is generally attributed to the estrogenic properties often seen with low doses of TAM. TAM takes approximately 4 weeks to reach effective steady-state levels, producing a window in which patients are exposed to suboptimal and potentially estrogenic concentrations of TAM (Buckely and Goa, 1989; Etienne *et al.*, 1989). These tumors are clearly not resistant to TAM, in either the pharmacologic or clinical context. Tumor flare should not be confused with the clinical TAM-stimulated resistance phenotype that may occur after prolonged TAM exposure and an initial TAM response.

Unlike tumor flare in previously untreated patients, evidence from MCF-7 human breast cancer xenografts suggests that some breast cancers may be initially growth inhibited by TAM, only to later become dependent on TAM for proliferation (Osborne *et al.*, 1987; Gottardis *et al.*, 1989; Connor *et al.*, 2001). These xenografts also retain the ability to be stimulated by estrogens (remain estrogen-dependent). Pharmacologically, this phenotype is not a resistance phenotype because the cells are clearly responding to the drug. However, a TAM-stimulated phenotype would represent clinical drug resistance because the nature of the response has changed in a manner that supports disease progression and would require a change in treatment. Acquired TAM dependence appears to reflect a switch in how the cells perceive TAM (as an ER agonist rather than antagonist). Several possible mechanisms may explain how this switch occurs in MCF-7 cells, including immunologic effects, ER mutations, and changes in growth factor or coregulator expression.

AIB1 and tamoxifen-stimulated growth as an antiestrogen resistance mechanism

AIB-1 (amplified in breast cancer-1; also known as SRC-3, RAC3, TRAM-1, pCIP, ACTR) is a steroid hormone receptor coactivator located on chromosome 20q12 (Anzick *et al.*, 1997) that has recently received attention as a possible contributor to antiestrogen responsiveness. AIB1 binds ER (Azorsa *et al.*, 2001), enhances the expression of cyclin D1 (Planas-Silva *et al.*, 2001), and exhibits somatic instability in some breast cancers (Dai *et al.*, 2002). AIB1's function as an ER coactivator produces increased transcriptional activation of ER (Anzick *et al.*, 1997). A novel AIB1 isoform (AIB-Δ3) has been recently reported that increases hormone and growth factor sensitivity (Reiter *et al.*, 2001) and increases the estrogenicity of 4-hydroxytamoxifen to a greater degree than wild-type AIB1 (Dr Anna Riegel, Georgetown University Medical School, personal communication). The mRNA for AIB-Δ3 was detected at levels higher than normal cells in 7/8 breast cancers (Reiter *et al.*, 2001).

The data in Table 4 show some of the characteristics of AIB1 amplification and expression in breast cancers. Most studies have explored either gene amplification (found in $<10\%$) or mRNA expression (reported in 10–64% of breast tumors). One study reported AIB1 protein expression as being above that seen in normal breast cells in approximately 10% of breast cancers by immunohistochemistry. Protein expression was detected at levels similar to or greater than those seen in normal breast cells in about 60% of ER+ tumors.

The association of AIB1 with ER status is difficult to determine from the small number of studies available. While AIB1 amplification has been associated with ER-positivity (Anzick *et al.*, 1997), increased AIB1 mRNA expression has been associated with ER-negativity (Bouras *et al.*, 2001). Similar proportions of detectable and undetectable AIB1 protein levels ($\sim$65%) were

Table 4 AIB1 amplification and expression in breast cancer (representative studies)

DNA amplification	*mRNA overexpression*	*Protein*	*Study*
10/105 (9.5%)	48/75 (64% relative to normal)	Not reported	Anzick *et al.* (1997)
56/1157 (4%) ER− 10/429 (2.3%) ER+ 45/769 (5.9%)	Not reported	Not reported	Bautista *et al.* (1998)
No data	26/83 (31%) High AIB1: ER+ 11/26 (42%) Low AIB1: ER+ 44/55 (80%)	Not reported	Bouras *et al.* (2001)
Not detected (0%)	3/23 (13%)	Not reported	Glaeser *et al.* (2001)
20/259 (7.7%)	Not reported	Not reported	Cuny *et al.* (2000)
Not reported	Not reported	4/41 (9.8% relative to normal) Present: ER+ 11/16 (69%) Absent: ER+ 12/21 (57%)	List *et al.* (2001)

found in ER+ tumors (12/21 had undetectable expression; 11/16 had detectable expression); no significant correlation between AIB1 and either ER or PR was found (List *et al.*, 2001).

Approximately 10% of all ER+ breast tumors may overexpress wild-type AIB1 protein (List *et al.*, 2001). It remains to be seen if this 10% is primarily comprised of TAM-stimulated tumors, and/or those tumors that exhibit AIB1 gene amplification. One recent study compared AIB1 (western) and erbB2 expression. The 5-year disease-free survival was lower in those tumors expressing high levels of both AIB1 and erbB2 when compared with those expressing high levels of AIB1 and low levels of erbB2. AIB1 and number of positive lymph nodes were also correlated with shorter disease-free survival in TAM-treated compared with untreated patients (Osborne *et al.*, 2003).

Overexpression of AIB1 and AIB1-Δ3 can confer a TAM-stimulated phenotype that should also be estrogen responsive (Dr Anna Riegel, Georgetown University Medical School, personal communication). The proportion of AIB1-overexpressing cells that are dependent upon this activity for survival/proliferation is unknown. The proportion of breast biopsies that respond mitogenically to both TAM and estradiol in short-term culture (4%; see below) suggests that up to one-half of AIB1-overexpressing tumors might be TAM-stimulated. Since these tumors are predicted to retain estrogen responsiveness, and may still synthesize estrogens, many likely retain responsiveness to aromatase inhibitors.

The AIB1-overexpressing phenotype is broadly similar to some MCF-7 TAM-stimulated xenograft models. Since wild-type MCF-7 cells already overexpress AIB1 (Azorsa *et al.*, 2001) and the AIB1-Δ3 (Reiter *et al.*, 2001), it is not surprising that selection against TAM might produce a TAM-stimulated phenotype. Indeed, this phenotype is already present in some MCF-7 cells without TAM selection (Dumont *et al.*, 1996). It remains to be seen whether this model is primarily driven by an overexpression of wild-type AIB1. Since the AIB1-Δ3 was identified in MCF-7 cells and is more potent, this isoform may also contribute to the phenotype of these xenografts and some human breast cancers. Indeed, this variant may prove to be more relevant in a broader context because of its ability to also affect growth factor signaling, an effect that could be important in both ER+ and ER− cells (Reiter *et al.*, 2001).

Clinical relevance of the tamoxifen-stimulated phenotype as an antiestrogen resistance mechanism

Direct evidence of a TAM-stimulated resistance phenotype in breast cancer patients is difficult to find. Indirect evidence may be found from studies that assessed the frequency of a TAM withdrawal response. These responses are evident when a tumor progressing on TAM regresses upon cessation of the TAM therapy. Recently, we completed an extensive review of the literature and found 241 cases in five studies where the authors looked specifically for evidence of TAM withdrawal responses (Clarke *et al.*, 2001b). Responses were assessed by relatively similar criteria and could be combined into three groups: complete response, partial response, and worse than partial response. Evidence was found for only 3/241 complete responses (1.2%) and 13/241 partial responses (5.4%). Over 90% of cases (225/241) experienced a worse than partial response to TAM withdrawal (225/241; 93.4%).

Since breast tumors are highly heterogeneous, the TAM-stimulated population may not be the dominant cell population in most tumors. Thus, elimination of the TAM-dependent/stimulated population may not be sufficient to induce a complete or partial clinical response because the bulk of the tumor is independent of any TAM-induced proliferation. In our evaluation of the literature, disease stabilization was the most common beneficial response to TAM withdrawal. Disease stabilization might indicate tumors that contain populations that are no longer growth-stimulated by TAM and/or a shift in the balance between cell loss/death and proliferation. Whatever the mechanisms, cells in these tumors are clearly not primarily dependent upon TAM for survival, since the great majority of patients (194/241; 80%) experienced disease progression upon TAM withdrawal even when disease stabilization is included as a beneficial response (Clarke *et al.*, 2001b).

These data imply that the majority of tumors in patients that progress on TAM treatment are not progressing because they have acquired a TAM-stimulated phenotype. Indeed, the responses reported for TAM withdrawal may be a mix of several possible mechanisms, including immunologic effects or other mechanisms not directly mediated through ER. Such indirect mechanisms can be largely eliminated in *in vitro* models. A study of 224 human breast cancer biopsies (153 ER+ and 71 ER−) used an *in vitro* approach to measure more directly the frequency of an ER-mediated, TAM- and/or estradiol-stimulated phenotype (Nomura *et al.*, 1990). Primary cultures of breast cancer biopsies were studied for the ability of TAM and estradiol to induce a mitogenic response *in vitro*. Only 11/153 (7%) of ER+ cultures exhibited a mitogenic response to TAM, a proportion surprisingly similar to the proportion (16/241; 6.6.%) of patients estimated to experience either a complete or partial response to TAM withdrawal (Clarke *et al.*, 2001b).

Of interest is the observation that only 6/11 of the TAM-stimulated tumors were also stimulated by estrogen (Nomura *et al.*, 1990). Thus, the TAM- and estradiol-stimulated phenotype, as expressed by some MCF-7 human breast cancer xenografts, reflected only 4% (6/153) of the phenotypes of the ER+ patient biopsies and only 50% of the TAM-stimulated phenotypes.

Together, these data imply that the TAM-stimulated phenotype is only one of several that produce clinical resistance. If up to 20% of initially hormone responsive cases become TAM-stimulated to some degree (estimate includes disease stabilization responses)—by whatever combination of cellular, molecular, and/or immunologic mechanisms this stimulation is conferred—a significant

number of women could be affected. Unfortunately, that still leaves the remaining 80% at risk of acquiring resistance through other mechanisms. From existing evidence, the TAM- and estradiol-stimulated phenotype exhibited by some MCF-7 xenografts may be a minor component of all TAM resistance phenotypes. Clearly, other antiestrogen resistance mechanisms exist, including antiestrogen unresponsiveness, and these remain to be identified and characterized.

Gene networks in estrogen receptor-mediated cell signaling in antiestrogen resistance

ERα expression is both necessary and sufficient to predict responsiveness to antiestrogens in a high proportion of breast tumors. Thus, antiestrogen-induced effects on ERα-mediated signaling are almost certainly of critical importance in effecting clinical responses in many tumors. Nonetheless, we still do not know the genes responsible for signaling to these effects, or whether the effects are primarily to induce cell death, repress cell survival, or a combination of both. As noted above, ER-independent events may also interact with ER-mediated signaling and this may be important in the broader context of a gene network that regulates antiestrogen responsiveness. Thus, estrogens and antiestrogens may differentially affect a gene network that contains some ER-regulated genes (Clarke and Brünner, 1995, 1996). More recently, this concept has been extended to incorporate the likely ability of integrated signals to induce apoptosis while concurrently blocking differentiation and proliferation (Clarke *et al*., 2001c). It is predicted that such a network would be affected by TAM in TAM-stimulated models by signaling through patterns similar to estradiol. In antiestrogen unresponsive cells, signaling through this network may use different signaling patterns and/or exhibit differential regulation/expression of some of the same genes affected by estradiol.

The concept of a network differs from that of a signal transduction pathway in that it requires the integration of several pathways, de-emphasizes the role of well-established single signal transduction pathways, and acknowledges the likelihood that few complex phenotypes are likely to be driven by a single gene/pathway (Clarke *et al*., 2001c). Owing to the plasticity of breast cancer phenotypes, as illustrated by the diversity of endocrine resistance phenotypes (Clarke and Brünner, 1995), the gene network concept seems reasonable. Considering signaling within the constraints of a single, linear pathway may be inappropriate. At best, such an approach is likely to produce an incomplete solution; at worst, it may be misleading.

Delineating the components of a signaling network for estrogens/antiestrogens may not be simple (Clarke and Brünner, 1996). ERs regulate gene expression through direct binding to EREs and direct interactions with other transcription factors including AP-1 and SP-1. The nature of ER activation is affected by ligand structure, and different ligands likely differentially affect the expression and function of the same members of any gene network. For example, raloxifene may regulate gene expression through novel pathways not affected by TAM or ICI 182,780 (Yang *et al*., 1996), and as noted above, antiestrogens differentially affect transcription when bound to ERα compared with ERβ. Regulation of the entire network or key components of the network may also be affected by ER-independent signaling, for example, as intracellular signals are perturbed by tumor–stromal cell interactions. Temporal and spatial organization of signaling components in a network is also critical. The likely complexity of network regulation has been described elsewhere (Clarke *et al*., 2001c).

Accepting the principle of a network is technically demanding because it requires experimental methods to evaluate concurrently the expression of multiple genes and informatic methods capable of integrating expression pattern analyses with functional information. Methods to obtain such high-dimensional data are well established and can be used to explore both the transcriptome and proteome of cells and tumors. However, data analysis methods for exploring gene expression microarray or two-dimensional gel electrophoresis data remain in their infancy and it may be several years before adequate methods become available and widely accepted.

A novel gene expression network in antiestrogen resistance (unresponsiveness)

We have begun to apply both proteome (Skaar *et al*., 1998) and transcriptome analyses (Ellis *et al*., 2002; Gu *et al*., 2002) to breast cancer cell lines, xenografts, and tumors to identify potentially important components of a large signaling network that may contribute to both estrogen independence and acquired antiestrogen resistance. Current informatic methods do not provide an easy way to uncover rapidly and correctly an entire signaling network. However, it should be possible to discover integral components of an overall network and eventually piece together these components to reveal the entire network's structure.

We first identified appropriate cellular models, derived adequate algorithms for data analysis, and began to explore the proteomes by two-dimensional gel electrophoresis and the transcriptomes by serial analysis of gene expression and gene expression microarrays. Remarkably few antiestrogen resistance models are available for study, and almost all are based on the MCF-7 human breast cancer cell line (reviewed in Clarke *et al*., 2001b). MCF-7 xenografts selected against TAM almost exclusively produce a TAM-stimulated phenotype, which may not be representative of the majority of human breast cancers (see below). Thus, we established several E2-independent but responsive breast cancer cell variants with differing antiestrogen response profiles.

MCF-7 cells were first selected for an ability to grow *in vivo* in ovariectomized athymic nude mice. The resulting variant (MCF7/MIII) is estrogen-independent

for growth both in cell culture and as xenografts (Clarke *et al*., 1989a), but retains responsiveness to antiestrogens; that is, it is estrogen-independent but has an antiestrogen responsive phenotype (Clarke *et al*., 1989a, b). We further selected these cells *in vivo* and found that repeated *in vivo* estrogen withdrawal, which generated the MCF7/LCC1 variant, did not substantially change the antiestrogen responsiveness of the cells (Brünner *et al*., 1993a). MCF7/LCC1 cells were then selected *in vitro* for resistance to 4-hydroxytamoxifen. The resulting MCF7/LCC2 cells are TAM-resistant but ICI 182,780 responsive (Brünner *et al*., 1993b). This phenotype predicted for the subsequent observation that patients responding to TAM, and then acquiring a TAM-resistant phenotype, have a high probability of retaining sensitivity to ICI 182,780 (Howell *et al*., 1995). In marked contrast, MCF7/LCC1 cells selected for resistance to ICI 182,780 (MCF7/LCC9 variant) acquire resistance to ICI 182,780 and crossresistance to TAM (Brünner *et al*., 1997). These models represent pharmacologic models of antiestrogen resistance in the context that they no longer respond to the growth inhibitory effects of antiestrogens. Models that reflect a switch to an antiestrogen-stimulated phenotype are described above.

By comparing the proteomes and transcriptomes of several of these MCF7/LCC variants, we have begun to identify what we believe is one component of a larger gene network that may regulate antiestrogen responsiveness. The relevance of this gene subset is already under intensive investigation in functional studies *in vitro* and *in vivo* and for its ability to improve prediction of antiestrogen responsiveness in breast cancer patients.

Candidate genes

The first goal in these studies was to identify differentially expressed genes and proteins that might contribute to acquired estrogen-independent and/or antiestrogen resistance. The data in Table 5 are adapted from our most recent study (Gu *et al*., 2002) and show the differential regulation of genes we use below to construct one component of a putative antiestrogen responsiveness signaling network. Functional studies of the interactions described in this network are currently in progress.

Table 5 Genes in a putative signaling network

Gene[a]	*Analysis*	*MCF7/LCC1 vs MCF7/LCC9*[b]
EGFR	Microarray	Twofold
EGR-1	Microarray	Threefold
IRF-1	Microarray	Twofold
NFκB	Microarray	0.5-fold
n-ras-related gene	SAGE	0.5-fold
Superoxide dismutase	Microarray	0.5-fold
TNFα	Microarray	Twofold
TNF-R1	Microarray	Twofold
X-box binding protein-1	SAGE	0.25-fold

[a]Links to the UniGene clusters for these and other genes from this study can be found at http://clarkelabs.georgetown.edu/Gu_et_al/Tables.htm. [b]Since the fold differences are relative to MCF7/LCC1 levels, genes upregulated in MCF7/LCC9 cells are expressed as a fraction

Comparing the MCF7/LCC1 and MCF-7 proteomes identified nucleophosmin (NPM) as being associated with estrogen independence (Skaar *et al*., 1998). NPM is a nucleolar, DNA/RNA-binding phosphoprotein (Wang *et al*., 1994; Herrera *et al*., 1995) that, when overexpressed in NIH 3T3 cells, produces a fully transformed phenotype (Kondo *et al*., 1997). Down-regulating NPM delays entry into mitosis (Jiang and Yung, 1999), perhaps reflecting its differential phosphorylation by key kinases: $p34^{cdc2}$ kinase (Peter *et al*., 1990), CDK2/cyclin E (Tokuyama *et al*., 2001), and protein kinase C (Beckmann *et al*., 1992). NPM binds the retinoblastoma protein to induce DNA polymerase α (Tchoudakova *et al*., 1999) and decreases susceptibility to butyrate-induced apoptosis through inducing telomerase activity (Liu *et al*., 1999). Overexpression of NPM is seen in colorectal (Nozawa *et al*., 1996) and prostate cancers (Bocker *et al*., 1995). NPM is E2-regulated in breast cancer cells (Brankin *et al*., 1998) and anti-NPM autoantibodies are readily detected in the sera of breast cancer patients (Brankin *et al*., 1998). NPM blocks the transcriptional activator functions of both YY1 (Inouye and Seto, 1994), which regulates β-casein production in the mammary gland (Raught *et al*., 1994), and the putative tumor suppressor gene interferon regulatory factor-1 (IRF-1). NPM regulates the stability and activation of p53 (Colombo *et al*., 2002), implicating its activities in p53-medated effects on apoptosis, and p53 is sequested in the cytosol of TAM-resistant MCF7/LCC2 cells (Lilling *et al*., 2002).

Exploring the MCF7/LCC1 and MCF7/LCC9 transcriptomes by SAGE identified several differentially expressed genes (Gu *et al*., 2002). We discuss here only the human X-box binding protein-1 (XBP-1) and the n-ras-related gene. XBP-1 is a member of the ATF/CREB transcription factor family that activates promoters containing CREs (Clauss *et al*., 1996). During liver regeneration, XBP-1 is associated with increased proliferation and reduced apoptosis (Reimold *et al*., 2000), implying a survival function that may explain the role of its overexpression in hepatocellular carcinomas (Kishimoto *et al*., 1998). Expressed within a cluster of genes associated with some ER + breast tumors (Perou *et al*., 2000), we have recently begun to explore XBP-1's role in normal and neoplastic breast cells.

The role of the n-ras-related gene is unclear. Ras expression is upregulated in many breast cancers (Clark and Der, 1995) and activates signaling through MAPKs that are also regulated by growth factors implicated in estrogen/antiestrogen responsiveness and mitogenesis (Dickson and Lippman, 1995; Clarke *et al*., 2001b; Santen *et al*., 2002). These MAPKs have been implicated in phosphorylating and activating ERs, an effect that could influence antiestrogen responsiveness (Clarke *et al*., 2001b; Santen *et al*., 2002). However, some recent studies suggest that MAPK's effects on ER do not

influence antiestrogen responsiveness (Atanaskova *et al.*, 2002).

Exploring the MCF7/LCC1 and MCF7/LCC9 transcriptomes by gene expression microarrays implicated several genes including IRF-1, nuclear factor-κB (NFκB), early growth response gene-1 (EGR-1), epidermal growth factor receptor (EGFR), and both tumor necrosis factor-alpha (TNFα) and its receptor TNF-R1 (Gu *et al.*, 2002). While initially identified as an interferon-induced gene, IRF-1 has now been implicated in regulating several critical cellular functions and is a putative tumor suppressor in some cancers (Tanaka *et al.*, 1994a, b; Yim *et al.*, 1997). IRF-1's tumor suppressor activities may be related to its ability to signal to apoptosis (Tanaka *et al.*, 1994a), which can occur in a p53-dependent or -independent manner (Tamura *et al.*, 1995; Tanaka *et al.*, 1996), with or without induction of $p21^{waf1/cip1}$ (Tanaka *et al.*, 1996) or $p27^{kip1}$ (Moro *et al.*, 2000), and through caspase-1 (Tamura *et al.*, 1995), -7 (Sanceau *et al.*, 2000) -8, (Suk *et al.*, 2001), and/or Fas-ligand (Chow *et al.*, 2000). Potentially related to these activities is the ability of SAPK p38, which is involved in signaling to apoptosis in response to stress, to activate IRF-1/interferon-stimulated reponse element binding (Varley and Dickson, 1999). Consistent with putative tumor suppressor activities, one small immunohistochemical study reports reduced IRF-1 expression in neoplastic *vs* normal human breast tissues (Doherty *et al.*, 2001).

The consequence of NFκB activation is cell context specific (Voegel *et al.*, 1996), but it is generally considered antiapoptotic in most cancer cells. Several aspects of normal mammary gland development appear dependent upon NFκB activity (Clarkson and Watson, 1999), likely reflecting its regulation by both estrogens and growth factors (Nakshatri *et al.*, 1997; Biswas *et al.*, 2000). Elevated NFκB activity arises early during neoplastic transformation in the rat mammary gland (Kim *et al.*, 2000). Widely expressed in human and rat mammary tumors (Sovak *et al.*, 1997; Cogswell *et al.*, 2000), upregulation of NFκB is associated with estrogen independence (Nakshatri *et al.*, 1997; Clarkson and Watson, 1999). NFκB is the only protein known to induce BRCA2 expression (Welcsh and King, 2001). Several excellent reviews on NFκB signaling are available (Bours *et al.*, 2000; Baldwin, 2001; Karin *et al.*, 2002).

EGR-1 is a transcription factor with proapoptotic activity (Das *et al.*, 2000) and is downregulated in DMBA-induced mammary adenocarcinomas in rats and mouse and human breast cancer cells (Huang *et al.*, 1997). c-myc is a major regulator of breast cancer proliferation and survival (Liao and Dickson, 2000) and is among the genes downregulated by EGR-1 (Hoffman *et al.*, 2002). EGR-1 also blocks NFκB function (Chapman and Perkins, 2000) and can stimulate apoptosis through cooperation with $p21^{waf1/cip1}$ and transactivation of p53 (Liu *et al.*, 1998). Superoxide dismutase (SOD) expression is increased in MCF7/LCC9 cells (Gu *et al.*, 2002) and in TAM-stimulated MCF-7 xenografts (Schiff *et al.*, 2000); SOD overexpression was previously implicated in resistance to TNFα (Zyad *et al.*, 1994). A TNFα-mediated pathway for signaling to apoptosis occurs in MCF-7 cells (Burow *et al.*, 1998; Egeblad and Jaattela, 2000), and measuring serum TNF concentrations may be a useful prognostic marker in breast cancer patients (Sheen-Chen *et al.*, 1997). Furthermore, IRF-1 expression is induced by TNFα in some cells (Mori *et al.*, 1999).

One component of a gene network

Using the data from our proteome and transcriptome studies and from other published studies, we have begun to construct a gene expression network for signaling in antiestrogen responsiveness (Figure 3). Studying a variant that is crossresistant to triphenylethylenes and steroidal antiestrogens (MCF7/LCC9) provided the opportunity to identify more broadly based resistance signaling than might be obtained from a study of TAM-only resistance (e.g., MCF7/LCC2 phenotype). The apparent consistency of the interactions among the

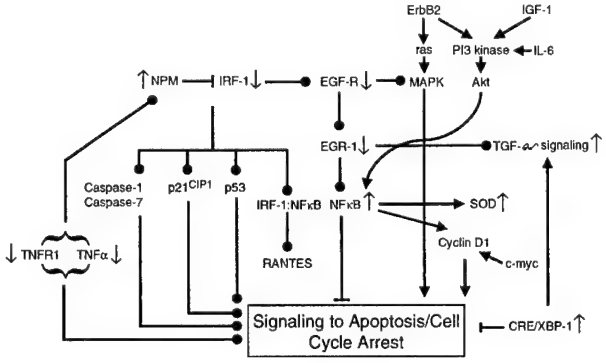

Figure 3 Part of a putative gene expression network constructed from the genes differentially expressed in MCF7/LCC9 cells (TAM and ICI 182,780 crossresistant) and their sensitive MCF7/LCC1 parent cells. Candidate genes from other studies are also incorporated into the network. Arrows represent those genes with altered expression, and the consequences of these changes are represented in the context of an antiestrogen-resistant phenotype. For example, the low levels of IRF-1 in MCF7/LCC9 cells are unable to induce EGFR, which remains low in these cells. Redundancy is evident; for example, the upregulation of NFκB and ras may compensate for low EGFR expression because they signal downstream of the EGFR's kinase activity. Signaling through this network component is expected to be different between sensitive and resistant cells and likely also different among some populations with the same phenotype. For example, not all resistant cells need to modify gene expression in the same pattern as apparently adopted by MCF7/LCC9 cells. Since ER-mediated effects are critical in antiestrogen-induced signals in sensitive cells, these cells may signal through the network component primarily comprising ER-regulated genes. While the interactions in this figure are consistent with published data, the network as represented is not intended to be complete and the regulation of some genes may be more complex than alluded to here. As we further evaluate signaling in these cells, we may identify additional components of this network. {} = receptor–ligand complex; ↑ = expression is increased; ↓ = expression is reduced; other arrows show direction of signal transduction; ⊥ = inhibition of indicated gene/function; —● = inability to induce substantially next signal or influence next event due to low/reduced expression/activity

relatively few genes incorporated into our network component is surprising. EGF-R induces expression of EGR-1 (Tsai *et al.*, 2000), and expression of both genes is lower in MCF7/LCC9 cells (Gu *et al.*, 2002). Since EGR-1 inhibits NFκB function (Chapman and Perkins, 2000), its low expression may contribute to the increased NFκB activity in these cells (Gu *et al.*, 2002). IRF-1 induces EGF-R mRNA (Rubinstein *et al.*, 1998), and IRF-1 levels are lower in MCF7/LCC9 cells (Gu *et al.*, 2002). IRF-1 is induced by TNFα/TNF-R1 (Mori *et al.*, 1999), both of which are also concurrently downregulated in MCF7/ LCC9 cells, perhaps explaining their lower IRF-1 levels. IRF-1 can act as a tumor suppressor and signal to apoptosis through both p53-dependent and -independent pathways (Taniguchi, 1997). These observations may reflect IRF-1's ability to affect caspase activity, since caspase activation and induction of apoptosis are implicated in affecting antiestrogen responsiveness (Mandlekar *et al.*, 2000a, b). Overexpression of caspase-1, which regulates apoptosis in normal mammary epithelial cells (Boudreau *et al.*, 1995), is known to be lethal in MCF-7 cells (Keane *et al.*, 1996). In these models, signaling through caspase-3 is unlikely because the gene is truncated in MCF-7 cells (Friedrich *et al.*, 2001); signaling through caspase-7 may dominate.

Interferons (IFNs) and TNF act synergistically to induce gene expression, an effect that appears driven by protein–protein interactions between IRF-1 and NFκB (Drew *et al.*, 1995; Neish *et al.*, 1995). IRF-1 can induce degradation of IκBα in some cells (Kirchoff *et al.*, 1999). IRF-1 : NFκB heterodimers affect expression of the ATF-2/jun (Escalante *et al.*, 1998), RANTES (Lee *et al.*, 2000), VCAM-1 (Neish *et al.*, 1995), IL-6 (Sanceau *et al.*, 1995), and MHC class 1 genes (Drew *et al.*, 1995). Altered AP-1 expression (includes jun) is implicated in the TAM-stimulated antiestrogen resistance phenotype (Schiff *et al.*, 2000), RANTES expression correlates with a poor prognosis (Luboshits *et al.*, 1999), VCAM-1 is involved in angiogenesis and metastasis in breast tumors (Byrne *et al.*, 2000), and autocrine production of IL-6 is associated with drug resistance in breast cancer cells (Conze *et al.*, 2001).

Unlike IRF-1, NPM expression is increased in MCF7/LCC9 cells compared with MCF7/LCC1 cells. NPM can function as an oncogene, its overexpression fully transforming NIH 3T3 cells in an assay for oncogenic potential (Kondo *et al.*, 1997). Levels of autoantibodies to NPM increase in patients 6 months prior to recurrence. Consistent with an antiestrogenic regulation of NPM, the levels of NPM autoantibodies are lower in breast cancer patients who received TAM (Brankin *et al.*, 1998). Concurrent upregulation of NPM and downregulation of IRF-1 suggest a novel signaling pathway in antiestrogen resistance. Both are estrogen-regulated genes in MCF-7 cells, IRF-1 expression being suppressed and that of NPM being induced (Skaar *et al.*, 1998, 2000). Through its direct binding to IRF-1, NPM inhibits the transcription regulatory activities of IRF-1 (Kondo *et al.*, 1997). Overexpression of NPM may eliminate the remaining IRF-1 activity, blocking its ability to initiate an apoptotic caspase cascade, and/or induce $p21^{waf/1cip1}$ (Coccia *et al.*, 2000) and cooperate with p53 in signaling to growth arrest and apoptosis (Tanaka *et al.*, 1994a, 1996).

XBP-1 acts through its ability to regulate genes containing CRE in their promoters (Clauss *et al.*, 1996). A cAMP-dependent pathway that inhibits IRF-1 transactivation has been described (Delgado *et al.*, 1999); XBP-1 activation of this pathway could suppress further the already low IRF-1 activity in some antiestrogen-resistant cells.

N-ras-induced signaling may also be important and implies an upregulation of ras-induced signaling in resistant cells. Such increased signaling may partly abrogate the need for growth factor-induced signaling through autocrine, paracrine, or intracrine stimulation (Clarke *et al.*, 2001b) because increased ras activation is downstream of several growth factor receptors implicated in breast cancer (Santen *et al.*, 2002). For example, cells may be capable of surviving when EGFR levels are reduced (Table 5) because loss of EGFR signaling is compensated by a downstream upregulation of ras-mediated signaling. Low IRF-1 expression may also contribute to the effects of ras signaling because IRF-1 induces lysyl oxidase (Sers *et al.*, 2002), which is implicated in reversing ras-induced malignant transformation (Contente *et al.*, 1999; Nozawa *et al.*, 1999).

Some of the genes we found have been implicated in antiestrogen resistance in other studies, most notable being EGF-R (Nicholson *et al.*, 2001) and its family member c-erbB2 (Kurokawa *et al.*, 2000; Welch and Clarke, 2002; Konecny *et al.*, 2003). AKT (Perez-Tenorio and Stal, 2002), c-myc (Carroll *et al.*, 2002), cyclin D1 (Varma and Conrad, 2002), p53, $p21^{waf1/cip1}$ (Fattman *et al.*, 1998), and AP-1 (Schiff *et al.*, 2000) may also contribute to antiestrogen responsiveness. We have incorporated some of this knowledge into the network in Figure 3, particularly where these genes may interact with those identified in our models. Several genes are thought to be downstream of signaling from growth factor receptors implicated in either phosphorylating/activating ER and/or inducing mitogenesis and affecting antiestrogen responsiveness (Chan *et al.*, 2001; Varma and Conrad, 2002). For example, the type I insulin-like growth factor receptor and c-erbB2 can activate AKT, which is often upstream of NFκB (Martin *et al.*, 2000). Several growth factors activate MAPK signaling to mitogenesis and signal through activation of ER. For simplicity, we have not shown all of these possible interactions in Figure 3.

Functional studies

We acknowledge that the gene network component in Figure 3 is somewhat speculative. Furthermore, it is unlikely to be regulated in the same way in TAM-stimulated models that perceive TAM as an estrogen. For example, in TAM-stimulated models, key network components could be perturbed in the same manner as expected with estradiol treatment.

One approach to assessing the likely validity of selected genes in our network component is to explore their functional activities and abilities to affect antiestrogen responsiveness in experimental models. We have begun several studies to further assess the likely functional relevance of our observations and support the gene network component in Figure 3. Transcriptional activation of XBP-1 and NFκB was studied using established promoter–reporter assays (CRE promoter–reporter assay for XBP-1). As predicted in the transcriptome analyses, increased basal transcription of both promoters was observed. Further studies showed that the ability of ICI 182,780 to inhibit NFκB activation is lost in the resistant cells. Preliminary data from our laboratory imply that the ability of antiestrogens to induce IRF-1 is also lost in resistant cells (Bouker *et al.*, 2002). Consistent with our earlier hypotheses (Clarke and Lippman, 1992), these data show significant changes in the endocrine regulation of some ER-regulated genes. We found no evidence for endocrine regulation of CRE activation in either responsive or resistant cells. However, resistant cells exhibit a significant fourfold increase in CRE activation, reflecting the fourfold increase in its expression predicted from the SAGE study. These observations suggest at least some general resistance mechanisms: an overexpression and loss of endocrine regulation of some genes that are ER-regulated in responsive cells, a downregulation and loss of endocrine regulation of some genes that are ER-regulated in responsive cells, and an upregulation of some endocrine unresponsive genes.

To study functional relevance further, the sensitivity of our variants to inhibition of NFκB activation by parthenolide was explored. Parthenolide, which is currently in early clinical trials, binds NFκB in a highly stereospecific manner (Garcia-Pineres *et al.*, 2001) and inhibits the IκB kinase repressor of NFκB (Hehner *et al.*, 1999; Patel *et al.*, 2000). We would expect that, if NFκB is providing a survival function, MCF7/LCC9 cells might be more dependent upon this activity. Indeed, MCF7/LCC9 cells are significantly more sensitive to growth inhibition by parthenolide than their MCF7/LCC1 parental cells (Gu *et al.*, 2002). Thus, some cells may survive antiestrogen exposure by upregulating estrogen-regulated survival factor(s) concurrent with the loss of their ER-mediated regulation. While we first need to confirm and extend these observations, parthenolide may prove useful in combination with Faslodex or other antiestrogens to either increase responsiveness and/or delay the appearance of resistant disease. Functional studies into the activities of the other genes in this network and investigations into their power to better predict antiestrogen responsiveness in patients are in progress.

Conclusions and future prospects

Acquired antiestrogen resistance likely comprises both true antiestrogen unresponsiveness (the major phenotype) and antiestrogen-stimulated growth (probably a minor phenotype). Several resistance mechanisms exist and, with the exception of loss of ER expression, these mechanisms may not be driven by a single gene or single signaling pathway. Consequently, we continue to develop the concept that an integrated gene network exists that allows cells a significant degree of plasticity in how they signal through this network (Clarke and Brünner, 1995, 1996; Clarke *et al.*, 2001c). More recently, we have begun to identify candidate genes in one component of this network and to explore their likely functional relevance in experimental models and ability to predict patient outcome. As we and others explore the transcriptomes and proteomes of experimental models and patient samples, additional components of this network may become apparent. Ultimately, understanding how breast cancer cells coordinate a response to antiestrogens, and overcome the growth inhibitory nature of the resulting signaling, may lead to better treatments and more powerful predictors of clinical response.

Some dietary components can modify the ability of TAM to inhibit the growth of ER+ and perhaps also ER− breast cancer cells. These dietary components might be those that alone are believed to affect recurrence of breast cancer. However, when consumed in combination with TAM, various dietary components could either potentiate or inhibit TAM's actions. Examples of unexpected findings are the studies of Ju *et al.* (2002) and Depypere *et al.* (2000), who showed that genistein or tangeretin prevents TAM from inhibiting growth of malignant breast cells. Currently, only a few published studies have examined the impact of nutrition on TAM's therapeutic effects, and it is likely that other dietary factors can modify TAM's ability to inhibit breast cancer growth.

The clinical use of antiestrogens, and TAM in particular, may change in the future. Data from some recent studies suggest that the current generation of aromatase inhibitors may be more effective than antiestrogens as first-line endocrine treatment for ER+ metastatic breast cancer and as adjuvant therapy for ER+ breast primaries (Buzdar and Howell, 2001; Ellis *et al.*, 2001). Nonetheless, the American Society of Clinical Oncology's Technology Assessment Working Group continues to recommend 5 years of adjuvant TAM as the standard therapy for women with ER+ breast cancer (Winer *et al.*, 2002). In terms of chemoprevention, the recommendations include the use of TAM *vs* participation in a clinical trial that involves the administration of raloxifene, any aromatase inhibitor, or any retinoid only within the context of chemoprevention (Chlebowski *et al.*, 2002).

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